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Received 15 June 2016; revised accepted 25 October 2016

doi: 10.18520/cs/v112/i08/1743-1749

Prevalence and multiple antibiotic resistance of *Vibrio coralliilyticus*, along the southwest coast of India

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Samples from two different estuaries (Cochin and Kumarakom) and a shrimp farm located along the southwest coast of India were analysed for the presence of *Vibrio* species. *V. coralliilyticus*, a global marine pathogen had high prevalence in all the three sources. The incidence of *V. coralliilyticus* was very high in the Cochin estuary (40%) when compared to the shrimp pond (20%) and Kumarakom estuary (19%). The susceptibility of *V. coralliilyticus* strains to 20 different antibiotics and their plasmid profiles were also checked. All the tested strains exhibited multiple antibiotic resistance, showing resistance towards 5–9 antibiotics tested. Resistance was shown towards amoxycillin, ampicillin, carbenicillin, oxytetracycline, trimethoprim, nitrofurantoin, furazolidone, sulphamethoxasole, erythromycin, while all the strains were sensitive to streptomycin, gentamicin, amikacin, netillin, tetracycline, chloramphenicol, cotrimoxasole, nalidixic acid, norfloxacin and ciprofloxacin. Multiple antibiotic resistance index varied from 0.25 to 0.55. Forty-three per cent of the isolates harboured 1–3 plasmids, with size ranging from 0.5 to 33 kb. Thus the present study demonstrates the high incidence, multiple antibiotic resistance and plasmid profiling of *V. coralliilyticus* from the southwest coast of India.

Keywords: Antibiotic resistance, estuaries, plasmid profiling, shrimp pond, *Vibrio coralliilyticus*.

VIBRIO, a Gram-negative halophile, is found naturally in shallow coastal waters to the deepest parts of the ocean. It is highly abundant in aquatic environments, including estuaries, marine coastal waters and sediments, and aquaculture settings worldwide and consists of more than 74 species[1]. Many *Vibrio* species are pathogenic to humans and animals. Hence, their prevalence and distribution in aquatic environments is of utmost public health importance. *Vibrio coralliilyticus* is a global marine pathogen that has been associated with coral disease from geographically distinct global regions. First isolated from diseased and bleaching corals off the coast of Zanzibar[2,3], this species has also been implicated in white syndrome disease outbreaks in the Indo-Pacific[4]. It causes fatal

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infections in a wide range of organisms, including unicellular algae, corals, oysters, shrimps, rainbow trout and flies during experimental infection assays[2,3,5–7]. The global distribution and the broad infectious potential of *V. coralliilyticus* to marine organisms highlight the need to study its distribution in the marine environment.

We studied the incidence of *Vibrio* sp. from two different estuaries, namely Cochin estuary which is greatly influenced by industrial, urban, human and hospital wastewater and the Kumarakom estuary which influenced by wastewater from rural farms, agricultural and human waste. Shrimp farms at Edavanakkadu region located along the Cochin estuary were also selected for our study. We also studied the antibiotic resistance pattern and plasmid profile of V. coralliilyt*icus* isolated from the study areas.

Samples were collected from ten stations in Cochin estuary (9°40′–10°12′N, 76°10′–76°30′E) and five stations in Kumarakom estuary (9°37′57″–9°38′21″N, 76°25′06″–76°25′11″E). Four traditional shrimp farms adjoining the Cochin backwaters and the feeder canal to these ponds were also selected for the study. The farms in this area are dependent on Cochin estuary for water.

Sediment and water samples were collected during pre-monsoon, monsoon and post-monsoon seasons for a period of one year using a Niskin water sampler. Then 500 ml of water sample from each station was filtered using 0.45 µm bacteriological filter. Pre-enrichment was done by transferring the filter to 100 ml alkaline peptone water and incubation at 37°C for 18–24 h. The sediment samples were collected using Van-Veen grab sampler. Sediments were analysed after making 10 fold dilutions in isotonic saline. For pre-enrichment, 1 ml of the diluted soil sample was transferred to 99 ml alkaline peptone water and incubated at 37°C for 18–24 h. A loopful of each enrichment broth was aseptically streaked onto thiosulphate citrate bile salt sucrose (TCBS; Himedia, India) agar plates and incubated at 37°C for 24 h. A few colonies were selected from the TCBS plates and stored in nutrient agar slants for further identification.

Preliminary screening of isolates was done based on oxidase test, Gram-staining, reactions on triple sugar iron agar (TSI) and O/F test. Genomic DNA of the presumptive isolates was extracted by phenol–chloroform method[8]. In order to avoid misidentification of other closely related genera as *Vibrio*, a universal primer set (Rflp-up 5′-TCCARAACATGGGCGCACAA-3′ and Rflp-rp 5′-ACGTTTTGYTCTTCGTTGTCRC-3′) was used to amplify a 1117-bp *groEL* gene fragment specific for *Vibrio*[9]. Template DNA (2 µg) was amplified in a 25 µl reaction volume containing 10 × PCR buffer, 25 mM $MgCl_2$, 2.5 mM dNTPs, 10 pM of each universal primer, and 0.5 U *Taq* polymerase. Cycling conditions included an initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation (94°C for 30 sec), annealing (69°C for 30 sec) and extension (72°C for 30 sec), with a final extension at 72°C for 7 min. All the isolates confirmed as *Vibrio* were further identified up to the species level using the dichotomous key provided by Noguerola and Blanch[10]. The following tests were used to characterize the vibrios: oxidase test, TSI, arginine dihydrolase test, ornithine decarboxylase, lysine decarboxylase test, growth in 0%, 3%, 6%, 8% and 10% NaCl, growth at 40°C, acid from sucrose, D-cellobiose, lactose, arabinose, D-mannose and D-mannitol, ONPG (ortho nitrophenyl-β-galactoside) test and Voges–Proskauer test.

Thirty isolates confirmed as *V. coralliilyticus* were tested for antibiotic sensitivity using the disc diffusion method[11]. Enriched bacterial cultures were aseptically swabbed onto Mueller–Hinton agar plates, the antibiotic-impregnated discs (Himedia, India) were placed on them and incubated overnight. Discs containing the following antibiotics were used: amoxicillin (Amx-10 µg), ampicillin (Amp-10 µg), amikacin (Ak-10 µg), carbenicillin (Cb-100 µg), sulphamethoxazole (Sm-300 µg), oxytetracycline (O-30 µg), chloramphenicol (C-30 µg), ciprofloxacin (Cip-5 µg), co-trimoxazole (Cot-25 µg), gentamicin (Gen-10 µg), netillin (Net-30 µg), nalidixic acid (Na-30 µg), norfloxacin (Nx-10 µg), nitrofurantoin (Nit-100 µg), enrofloxacin (Ex-5 µg), erythromycin (E-15 µg), streptomycin (S-10 µg), chloramphenicol (C-30 µg), trimethoprim (Tr-5 µg), tetracycline (Te-30 µg) and furazolidone (Fr-50 µg). The antibiotics belonged to 10 different classes according to their chemical structure.

The diameter of the zone of inhibition was measured after incubation. The results were interpreted following the recommendations of the Clinical Laboratory Standards Institute (CLSI, USA). Multiple antibiotic resistance (MAR) index of an isolate is the ratio between the number of antibiotics to which an isolate is resistant and the total number of antibiotics to which the isolate has been exposed[12].

Bacterial strains were cultured in 10 ml Luria–Bertani broth (Himedia, India) and incubated overnight at 37°C in a shaker incubator (200 rpm; Scigenics Biotech, India).

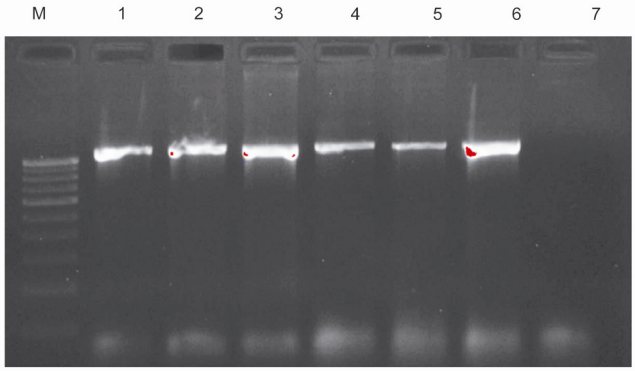

Figure 1. PCR-amplified *Vibrio*-specific *groEL* gene fragment. Lane M, 100 bp ladder; lanes 2–6, Positive isolates showing 1117 bp PCR product and lane 7, Negative control (non-*Vibrio* species).

Table 1. Resistance and susceptibility profiles of *V. coralliilyticus* isolates against 20 different antibiotics

Mode of action	Protein synthesis inhibition								Folate pathway inhibitors			Cellwall synthesis inhibition			DNA synthesis inhibition				DNA damage	
Class	Macrolides	Amino glycosides				Tetracycline		Phenicols	Pyrimidine	Sulphonamides		B lactams			Quinolones				Nitrofurans	
Antibiotics	E*	S	Gen	Net	Ak	Te	O	C	Tr	Sm	Cot	Amp	Amx	Cb	Na	Cip	Nx	Ex	Nit	Fr
W180 (Kumarakom)	S	S	S	S	S	S	S	S	R	R	S	R	R	S	S	S	S	S	S	R
W199 (Kumarakom)	S	S	S	S	S	S	S	S	S	R	S	R	R	R	S	S	S	R	S	S
W143 (Kumarakom)	S	S	S	S	S	S	R	S	S	R	S	R	R	R	S	S	S	R	R	R
W569 (Kumarakom)	S	S	S	S	S	S	R	S	R	R	S	R	R	R	S	S	S	R	R	R
1W7 (Cochin)	S	S	S	S	S	S	S	S	S	S	S	R	R	S	S	S	S	R	S	R
1W2 (Cochin)	S	S	S	S	S	S	R	S	R	S	S	R	R	S	S	S	S	R	R	R
1W6 (Cochin)	S	S	S	S	S	S	R	S	R	S	S	R	R	R	S	S	S	R	R	R
4W2 (Cochin)	S	S	S	S	S	S	R	S	S	R	S	R	R	S	S	S	S	R	R	R
3W9 (Cochin)	S	S	S	S	S	S	S	S	R	R	S	R	R	R	S	S	S	R	R	R
10W2 (Cochin)	S	S	S	S	S	S	S	S	R	R	S	R	R	R	S	S	S	R	R	R
3S3 (Cochin)	S	S	S	S	S	S	S	S	S	R	S	R	R	R	S	S	S	R	R	R
10W4 (Cochin)	S	S	S	S	S	S	S	S	R	R	S	R	R	R	S	S	S	R	R	R
7W5 (Cochin)	S	S	S	S	S	S	S	S	R	R	S	R	R	R	S	S	S	R	R	R
3W4 (Cochin)	R	S	S	S	S	S	S	S	R	R	S	R	R	R	S	S	S	R	R	R
5W1 (Cochin)	R	S	S	S	S	S	R	S	S	R	S	R	R	S	S	S	S	R	R	R
9S4 (Cochin)	R	S	S	S	S	S	R	S	R	R	S	R	R	S	S	S	S	R	R	R
10W1 (Cochin)	S	S	S	S	S	S	S	S	S	R	S	R	R	S	S	S	S	R	R	R
6S1 (Cochin)	R	S	S	S	S	S	S	S	R	R	S	R	R	S	S	S	S	R	R	R
6S4 (Cochin)	S	S	S	S	S	S	S	S	S	R	S	R	R	S	S	S	S	R	R	R
M7S3 (Cochin)	S	S	S	S	S	S	S	S	S	S	S	R	R	R	S	S	S	R	R	R
M8W2 (Cochin)	R	S	S	S	S	S	S	S	S	R	S	R	R	R	S	S	S	R	R	R
PM1S5 (Cochin)	R	S	S	S	S	S	S	S	S	R	S	R	R	R	S	S	S	R	R	R
PM6W4 (Cochin)	R	S	S	S	S	S	R	S	S	R	S	R	R	S	S	S	S	R	R	R
PM3W5 (Cochin)	R	S	S	S	S	S	S	S	S	R	S	R	R	S	S	S	S	S	R	R
AWV20 (Shrimp pond)	S	S	S	S	S	S	S	S	R	R	S	R	R	S	S	S	S	S	R	R
MaySV2 (Shrimp pond)	S	S	S	S	S	S	S	S	R	S	S	R	R	S	S	S	S	S	R	R
AWA24 (Shrimp pond)	S	S	S	S	S	S	R	S	R	R	S	R	R	R	S	S	S	R	R	R
MWE16 (Shrimp pond)	S	S	S	S	S	S	R	S	R	R	S	R	S	S	S	S	S	R	R	R
AWV6 (Shrimp pond)	S	S	S	S	S	S	R	S	S	S	S	R	R	S	S	S	S	R	R	R
ASA18 (Shrimp pond)	S	S	S	S	S	S	S	S	S	R	S	S	S	R	S	S	S	R	R	R

This culture was used for plasmid extraction following the alkali lysis method[8]. Electrophoresis was performed in 0.8% agarose gel (Himedia, India) 1% (w/v) in 1X TBE buffer (Himedia, India). Electrophoretic separation was peformed at 75 V for 2 h. In order to analyse the size of the plasmids, a molecular weight marker (supercoiled DNA ladder, Himedia, India) was also included. The gels were visualized under UV transilluminator using Gel Documentation System (GelDoc EZ imager, Bio-Rad, USA).

After preliminary screening, the isolates were confirmed as *Vibrio* by PCR amplification of *groEL* gene. Only the isolates belonging to *Vibrio* amplified the 1117 bp *groEL* gene fragment (Figure 1). This is the most conserved fragment of the *groEL* gene. None of the non-*Vibrio* species amplified the gene fragment. This prevented initial misidentification of closely related members of other genera as *Vibrio*.

When the isolates from the sampling areas were subjected to species-level identification using the standard dichotomous key, we observed that *V. coralliilyticus* was the most predominant species from all the three sources. All other *Vibrio* species showed lesser incidence when compared to this species (data not given). About 40% of the isolates from Cochin estuary, 20% from the shrimp pond and 19% from Kumarakom estuary belonged to *V. coralliilyticus* species. Bubble plots were constructed using PRIMER6 software to show the abundance of the species at various stations (Figure 2). Among the various stations in Cochin estuary, CS1 (marine science jetty) and in the shrimp pond, SP1 (feeder canal which brings water to the farm from Cochin backwaters) showed highest abundance of the species, whereas in Kumarakom estuary the species was found to have an equal distribution in all the five stations.

Of the total 30 *V. coralliilyticus* strains tested for antibiotic susceptibility towards 20 different antibiotics, all of them exhibited multiple drug resistance. Table 1 shows resistance and susceptibility patterns of the strains. All the strains were sensitive to 10 antibiotics falling under the following structural classes: aminoglycoside (streptomycin, netillin, amikacin and gentamycin), quinolones (nalidixic acid, ciprofloxacin and norfloxacin), tetracycline (tetracycline), phenicols (chloramphenicol) and sulphonamide (cotrimoxasole). Resistance was shown towards beta lactams (amoxicillin, ampicillin and carbenicillin), tetracycline (oxytetracycline), pyrimidine (trimethoprim), ntirofurans (nitrofurantoin and furazolidone), sulphonamides (sulphamethoxasole), quinolones (enrofloxacin) and macrolides (erythromycin). Figure 3 shows the percentage resistance of the strains to different antibiotics. The MAR index of the strains varied from 0.25 to 0.55 (Table 2).

The 30 multiple antibiotic-resistant *V. coralliilyticus* strains were screened for the presence of plasmids. Forty-three per cent of the strains harboured plasmids of size ranging from 0.5 to 33 kb (Table 2). Among 13 strains harbouring plasmids, 12 were from Cochin estuary and one from the shrimp pond. None of the isolates from Kumarakom estuary revealed the presence of plasmids. No correlation could be observed between antibiotic resistance and presence of plasmids.

In the present study, *V. coralliilyticus* showed high prevalence in Cochin and Kumarakom estuaries and the adjoining shrimp farm along the southwest coast of India. *V. coralliilyticus* has been previously isolated from marine organisms in the Atlantic[2,13], Indian Ocean[2], and Pacific Oceans[14,15], Mediterranean Sea[16] and Red Sea[2]. Although it is uncertain whether this organism is a primary or opportunistic coral pathogen, evidence strongly

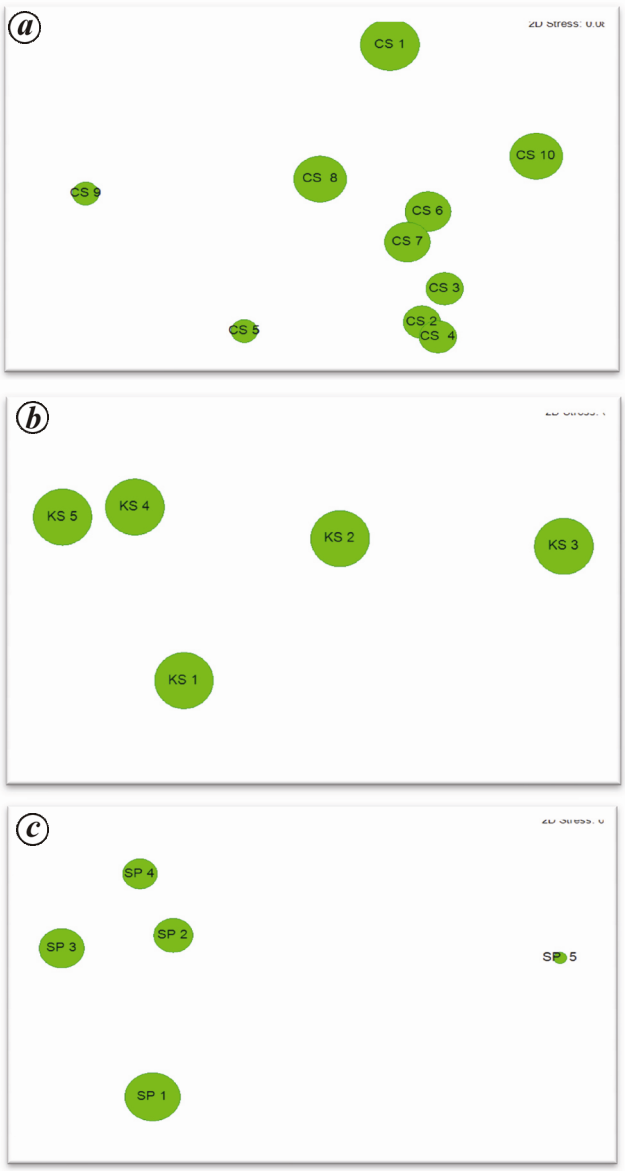

Figure 2. Bubble plots showing abundance of *Vibrio coralliilyticus* in (***a***) Cochin estuary, (***b***) Kumarakom estuary and (***c***) Shrimp pond.

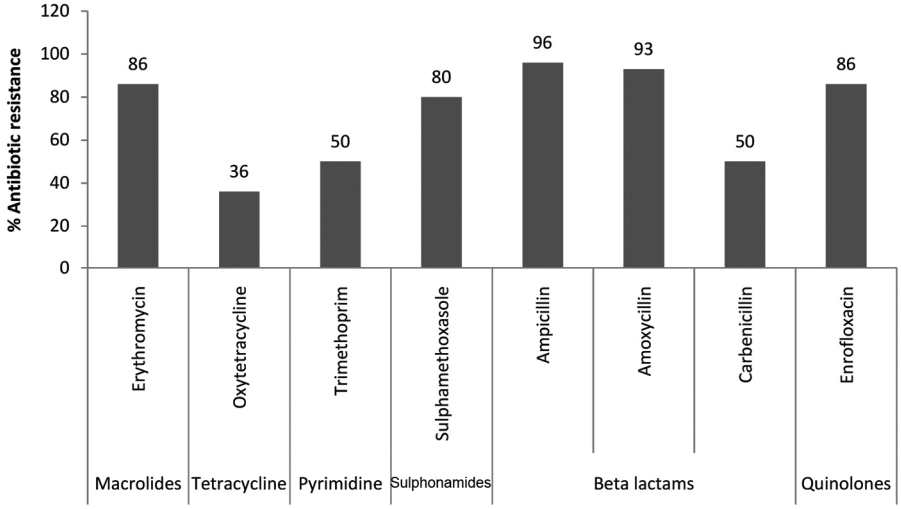

Figure 3. Percentage antibiotic resistance of *Vibrio coralliilyticus* isolates.

Table 2. Multiple antibiotic resistance index and plasmid profile of *V. coralliilyticus* isolates

Isolated strain	MAR index	Plasmid
W180 (Kumarakom)	0.25	None detected
W199 (Kumarakom)	0.30	None detected
W143 (Kumarakom)	0.40	None detected
W569 (Kumarakom)	0.55	None detected
1W7 (Cochin)	0.25	None detected
1W2 (Cochin)	0.35	None detected
1W6 (Cochin)	0.40	None detected
4W2 (Cochin)	0.35	Two (33 kb, 1 kb)
3W9 (Cochin)	0.40	None detected
10W2 (Cochin)	0.40	Two (33 kb, 8.9 kb)
3S3 (Cochin)	0.30	Two (33 kb, 8.9 kb)
10W4 (Cochin)	0.40	One (33 kb)
7W5 (Cochin)	0.40	None detected
3W4 (Cochin)	0.50	Two (33 kb, 8.9 kb)
5W1 (Cochin)	0.40	None detected
9S4 (Cochin)	0.50	None detected
10W1 (Cochin)	0.30	One (33 kb)
6S1 (Cochin)	0.40	One (33 kb)
6S4 (Cochin)	0.30	One (33 kb)
M7S3 (Cochin)	0.35	Two (33 kb, 4 kb)
M8W2 (Cochin)	0.40	One (33 kb)
PM1S5 (Cochin)	0.40	Four (33 kb, 2 kb, 1 kb, 0.5 kb)
PM6W4 (Cochin)	0.30	None detected
PM3W5 (Cochin)	0.30	Four (33 kb,7 kb, 8 kb, 9 kb)
AWV20 (Shrimp pond)	0.45	None detected
MSV2 (Shrimp pond)	0.25	None detected
AWA24 (Shrimp pond)	0.45	One (33 kb)
MWE16 (Shrimp pond)	0.40	None detected
AWV6 (Shrimp pond)	0.30	None detected
ASA18 (Shrimp pond)	0.30	None detected

suggests that this endemic member of the global coral holobionts[17] has a role in coral disease[18]. Addition of *V. coralliilyticus* supernatants to coral juveniles causes not only inhibition of photosynthetic activity, as with the *in vitro Symbiodinium* cells, but also loss of *Symbiodinium* cells from the coral juveniles and rapid onset of tissue lesions followed by complete mortality of the juvenile colony[19]. Previous reports show that *V. coralliilyticus* displays a tightly temperature-related virulence. It is found to be avirulent at temperatures ≤24°C and is considered virulent at temperatures above 24.5°C (ref. 3). At temperatures of 25°C and above, this Gram negative bacterium was found in high concentrations in the bleached coral *Pocillopora damicornis* (collected from the Red Sea and Indian Ocean)[2,3,20]. Reported virulence mechanisms of *V. coralliilyticus* include chemotaxis via flagella-mediated motility[21] and the production of an extracellular protease whose activity increases above 25°C (refs 2 and 19). We could observe that the temperature of all stations was above 24.5°C throughout the study period. This is to be taken into consideration since an environment with such a favourable temperature is a trigger for rapid proliferation and expression of virulence factors by *V. coralliilyticus* and other pathogenic vibrios. Consequently, it may infect the marine organisms present in the study area.

Multi-antibiotic resistance has been observed in bacteria from aquaculture environments, which is often associated with the use of some drugs[22]. Recently, higher frequency of drug-resistant *Vibrio* has been reported[23,24]. The differences in percentage of bacterial resistance to various antibiotics reflect the history of antibiotic application, and hence bacterial drug resistance can be applied as an indicator of antibiotic application in a locality[25]. All the isolates in the present study were sensitive to aminoglycosides (streptomycin, netillin, amikacin, gentamycin), quinolones (nalidixic acid, ciprofloxacin and norfloxacin), tetracycline (tetracycline), phenicols (chloramphenicol) and sulphonamide (cotrimoxasole) suggesting low contamination with these antibiotics in the sampling sites. High resistance of the strains towards

β-lactams (amoxicillin, ampicillin and carbenicillin), tetracycline (oxytetracycline), pyrimidine (trimethoprim), nirofurans (nitrofurantoin, furazolidone), sulphonamides (sulphamethoxasole) and macrolides (erythromycin) can be assumed to be due to the influence of anthropogenic activities (hospital waste) or wide application of these antibiotics in aquaculture settings adjoining the study sites. It was noted that none of the isolates from Kumarakom estuary and the shrimp pond showed resistance towards erythromycin, suggesting low contamination of the sites with this antibiotic. Resistance towards β-lactam antibiotics has been previously reported in *V. coralliilyticus*[13] and other vibrios from different sources[26,27]. Contrary to our findings, previous studies reported sensitivity of *V. coralliilyticus* strains towards nitrofurantoin and trimethoprim, and resistance to nalidixic acid, ciprofloxacin, streptomycin, amikacin, gentamicin and tetracycline[13]. Almost all the isolates tested showed MAR index-up to 0.55. Isolates with MAR index >2.0 are often known to originate from higher-risk sources of contamination such as humans, commercial poultry farms, swine and dairy cattle where this antibiotics can be used. High incidence of multiple drug-resistant isolates is a serious issue since they can act as potential sources of drug resistance genes in the environment. These genes enter the pathogens from non-pathogens through horizontal gene transfer mechanism. This leads to transfer of drug resistance characters to extreme human pathogens present in the environment. Thus, studies on genetic elements like plasmids, transposons and integrons associated with antibiotic resistance in microorganisms become important.

The present study provides an analysis of plasmid profile of *V. coralliilyticus* from South India. All the isolates which harboured plasmids had a common 33 kb plasmid along with other smaller plasmids, which is similar to the results of Zhang *et al.*[28] showing the presence of >30 kb plasmids in environmental *Vibrio* isolates. Bacterial antibiotic resistance patterns are usually associated with the presence of large plasmids with the ability for conjugation. Most conjugative R plasmids are usually as big as 30 kb (ref. 29). Even though no visible correlation was observed between antibiotic resistance pattern and presence of plasmids in the study, further plasmid curing experiments need to be performed to confirm the same. The plasmids profiles in vibrios have been previously studied in some species such as *V. parahaemolyticus*[30]; *V. ordalii*[31], *V. vulnificus*[32] and *V. salmonicida*[33], and most extensively in *V. anguillarum*, where a high diversity of profiles was observed[15,34]. Presence of plasmids in *Vibrio* species of both polluted and pristine environments may be ecologically important to the survival of these bacteria in the environment[28].

The occurrence of multiple drug resistance bacteria from the present study area emphasizes the importance of surveillance of drug susceptibilities of halophilic *Vibrios*. Hence, continuous monitoring of prevalence and antimicrobial susceptibility of vibrios is needed from the study sites.

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Received 1 June 2015; revised accepted 28 October 2016

doi: 10.18520/cs/v112/i08/1749-1755

Opportunistic predatory behaviour in *Duttaphrynus melanostictus* (Schneider, 1799) tadpoles

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We report *in situ* and *ex situ* observations on inter- and intra-specific predatory behaviour in tadpoles of the common Asian toad *Duttaphrynus melanostictus*. *In situ D. melanostictus* tadpoles feed on conspecific eggs, tadpoles of various developmental stages and adult carrion as well as dead heterospecific (*Fejervarya orissaensis* and *Euphlyctis cyanophlyctis)* tadpoles. Predation of weak, feebly swimming larvae and metamorphs in seminatural habitats under optimum conditions seems to be an opportunistic behaviour and diet enrichment, which needs additional support. Our observations support earlier reports indicating gradual desiccation, food shortage, competition and density as the probable factors of predation in temporary habitats.

Keywords: *Duttaphrynus melanostictus*, predation, tadpole, scavenger

PREDATORY behaviour is a widespread phenomenon in the animal kingdom. It is well documented among several anuran tadpoles (larvae) which demonstrate predatory interactions, including oophagy, cannibalism and necrophagy[1]. It has been recorded in those species that breed in temporary ponds, ephemeral pools or puddles where they occur in high densities and are deprived of food[2]. Cannibalism in natural or experimental conditions is quite common among tadpoles[3,4]. Tadpoles of some species feed on conspecific eggs or tadpoles[5–7], while others prey upon heterospecific tadpoles[8,9]. Factors such as food and space availability, microenvironment and mineral nutrients essential for metamorphosis shape the status of cannibalism in anurans[10]. Most cases of cannibalism involve oophagy[3,4,11,12], but occurrence of tadpole–tadpole cannibalism typically involves predation on different life stages[1].

The common Asian toad *Duttaphrynus melanostictus* is widely distributed in South and Southeast Asia; it breeds in both lentic (temporary and permanent pools) and lotic habitats (slow-flowing streams and canals). These tadpoles are gregarious and depending upon the circumstances, they may live as members of kin and/or mixed groups until metamorphosis[13]. We report predatory

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