

Original Research Article

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Prevalence and Characterization of Antibiotic Resistance Associated with *Escherichia coli* Isolated from Cultured *Penaeus vannamei* from Maharashtra, India

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ABSTRACT

Keywords

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This study examined the prevalence of antimicrobial resistance associated with *E. coli* which is an indicator organism of contamination in shrimp ponds of Vannamei culture. A total of 173 isolates were isolated from 60 shrimp samples of selected areas of Maharashtra and were evaluated for isolation, identification and antibiotic sensitivity pattern for *E. coli*. Out of 173 isolates 67 isolates were found to be *E. coli* positive which is of 39.2 %. The antimicrobial resistance profile of the tested *Escherichia coli* isolates to 12 different antibiotics revealed that all 67 isolates were sensitive to Furazolidone, Nalidixic acid, Neomycin, Nitrofurantoin, Norfloxacin, Trimethoprim whereas, the isolates showed resistant to ampicillin (75%) and Erythromycin (61%). The molecular characterization of *erm* genes which is predominantly associated with resistance among *Enterococcus* spp showed that 61.2% of the isolated *E. coli* were positive for *ermB* gene, whereas 42.5% of the isolates showed positive for *ermC* gene.

Introduction

Indian shrimp aquaculture showed a tremendous growth during the past decade, mainly due to the intensification in the shrimp farming practices. According to USDA, in 2015, India's export of shrimp was 382,959 tons which is about 9.8percent growth from the previous year (USDA, 2017). Moreover, India's cultured shrimp production in 2014-15

was about 426,500 MT, with a 30 percent growth from the previous year. This intensification leads to the emergence of several bacterial diseases with increasing use of antimicrobials for their control. Research indicates that the 70-80% of the drugs used in aquaculture ends up in the environment as large amount of those antimicrobial were used as therapeutic and prophylactic. Consequently, they may contribute to the major problem of

development of drug resistance among shrimp pathogens. Antimicrobial resistant bacteria also known as 'superbugs' are a global concern as defined by WHO, the most urgent resistant trend. AMR risks the gains of Millennium Development Goals and endangers the Sustainable Development Goals. India has set highest record in shrimp entry line rejections in 2016. This rising rejection indicates the widespread use of antimicrobials in aquaculture for therapeutic and non-therapeutic uses in India (press release by the MPEDA). Accounting the usage of the antibiotics in pond management, about 27% used them to prevent or treat viral diseases. According to MPEDA report, in 2012-2015, about 17 cases of exported shrimp products were reported for the presence of prohibited substances such as nitrofurantoin metabolite (furazolidone), nitrofurazone and chloramphenicol. In recent studies in various countries, the prevalence of antimicrobial-resistant bacteria in shrimp farms and shrimp products has been reported.

To analyse the antimicrobial resistance status of the enteric microflora of both fish and shrimp farms, *E. coli* also has been introduced as an indicator species in surveillance programs nowadays. This enteric bacteria in fish can also pose public health hazard (Rao and Gupta, 1978) as many studies have insisted that coliforms are not normal flora of fish and shellfish and they are not pathogenic to humans, but their presence indicates a higher risk of pathogens being present, which will affect humans more than they do aquatic organisms (Rudolfs *et al.*, 1950; Cuelin, 1962; Rao *et al.*, 1968; Cohen and Shuval, 1973; Evison and James, 1973; Lekshmy *et al.*, 2014). The drug-resistant *E. coli* strains on farms might come from a variety of sources, including workers' hands, animal waste, shrimp feed, and water. However, little is known about the drug resistance and the genetic mechanisms of resistance in *E. coli*

isolates from shrimps in India as most of the studies have focused on the human pathogens. An attempt is made in the present study to analyse the incidence of *E. coli* in the shrimp farms and its antibiotic resistance has been tested with 12 antibiotics.

Materials and Methods

The present study was conducted at the Department of Aquatic Animal Health, Central Institute of Fisheries Education, Mumbai, India. A total of 60 live shrimp samples were collected for the present study from selected fish farms of Thane, Naigoan and Bhayandar region of Maharashtra. Shrimps were anaesthetized by dipping in ice slurry and dissected in sterile condition. Stomach and intestine were removed and pooled separately by homogenising in 9ml of 2% saline solution using pestle and mortar. Each organ was then run through a series of dilution of 2% saline beginning at 1:10 to 1:10000 (1g tissue/ 9ml 2% saline solution). The samples were subjected to bacterial culture examination using standard microbiology procedures. Each of the shrimp samples were transferred to 10 ml of nutrient broth and incubated at 37°C for 22-24 hours to resuscitate the organisms. A loopful of sample was then streaked on nutrient agar plates in progressive fashion in order to obtain isolated colonies of bacteria. These petri plates were incubated for 24 hrs at 37°C. After 24 hrs of incubation these isolated colonies were cultured on eosin methylene blue agar (EMB) plates for isolation of *Escherichia coli*. Re-inoculation of the selected colonies were continued until pure bacterial colonies were obtained. Pure isolated bacteria were preserved for further analysis using agar slants. The growth was examined for the colonial morphology and pigmentation, and different types of colonies were sub-cultured on separate nutrient agar plates in order to obtain a pure culture.

The confirmation of the isolates as *Escherichia coli* were done using Gram's staining, Catalase test, Oxidase test, Coagulase test and 12 biochemical tests for *Escherichia coli*. Antibiotic sensitivity test (AST) was conducted as per the disc diffusion method prescribed by Kirby *et al.*, (1966). Spread the overnight culture on Mueller-Hinton agar plate surface evenly using sterile cotton swabs. Place the filter paper discs containing standardized quantities of antimicrobial drug onto the agar surface within 15 minutes of spreading the culture. These petri plates were incubated at 37^o C for 18-24 hrs. To conduct antibiotic sensitivity test, 12 antibiotic discs were selected viz., Ampicillin (10mcg), Aztreonam (30mcg), Cephalothin (30mcg), Chloramphenicol (30mcg), Erythromycin (15mcg), Furazolidone (30mg), Nalidixic acid (30mcg), Neomycin (30mcg), Nitrofurantoin (300mcg), Norfloxacin (10mcg), Trimethoprim (300mcg) and Tetracycline (30mcg).

Bacterial cultures on nutrient agar slants were used to extract crude DNA, by the use of boiling cell lysis extraction method. A single colony of bacterial cells was suspended in 50 µl of distilled water and vortexed shortly. The suspension was heated at 99°C for 5 min and then was chilled in ice for 10 min. The cell debris was pelleted by centrifugation at 13,400 g for 2 min and 5 µl of clear supernatant was used as the DNA template in a PCR. The PCR was done using previously described primers specific for *erm B* and *ermC* genes (Nguyen *et al.*, 2009). Table 1 shows the details of the primers used in this study. The PCR products were then separated using electrophoresis on 1.5% agarose gel, and visualised under UV by staining with ethidium bromide.

Results and Discussion

Out of 60 shrimp samples, 173 bacterial isolates were isolated and characterized with

standardized laboratory methods. Of the 173 isolates 67 (39%) were found positive for *Escherichia coli*. Faridullah *et al.*, reported 60% prevalence of positive isolates of *E. coli* from shrimp samples of 30 numbers in Bangladesh (Faridullah *et al.*, 2016). Roy *et al.*, isolated 12 *E. coli* samples in 48 samples of shrimp intestine farmed shrimp (Roy *et al.*, 2013). Moreover, Sugumar *et al.*, (2001) have reported increased coliform loads in shrimp farming system. This study reveals that all the isolates that were gram negative, oxidase negative, catalase positive. All *Escherichia coli* strains gave metallic sheen on EMB agar. Biochemical test proposed by Oliver *et al.*, (1992), and Alsina *et al.*, (1994) were done for confirmation of *Escherichia coli*.

The isolates showing positive reaction for the Nitrate reduction test, Methyl red test, Indole test, ONPG test, Lysine utilization test, Lactose, Glucose, Sucrose and Sorbitol sugar tests while negative with Voges Proskauer's test and Citrate utilization test were confirmed as *Escherichia coli* (Koneman *et al.*, 2001) which is described in the Table 2.

The isolated 173 bacterial colonies were subjected to antimicrobial susceptibility testing proposed by Kirby & Bauer (1966). The reported commonly used antibiotics in shrimp and fish aquaculture by Costa *et al.*, 2015, Holmstrom *et al.*, 2003, Almada *et al.*, 2012 were used for this study. Vieria *et al.*, in his study confirmed that more than 90.5% of strains of *Escherichia coli* showed a variety of resistance profiles to the tested antibiotics (Vieria *et al.*, 2010). In the present study, out of the 67 tested isolates, most of the isolates were resistant to ampicillin and erythromycin antibiotics. The antimicrobial resistance profile of the tested isolates *Escherichia coli* to different antibiotics revealed that out of the 67 isolates, 50 isolates (75%) were resistant to Ampicillin, 35 isolates (53%) were resistant to Cephalothin, 23 isolate (35%) were resistant to

Aztreonam and 41 isolates (61%) were resistant to Erythromycin. In study conducted by Changkaew the resistant isolates were found in shrimp is of 29.6% which is 8 of 27 isolates, and high resistance rates were found for tetracycline, ampicillin, and trimethoprim (Changkaew, *et al.*, 2005). The Table 3 gives the brief details on the antimicrobial resistant profile of those isolated *E.coli*. Intermediate resistance were observed against five antibiotics – Ampicillin, Aztreonam,

Cephalothin and Erythromycin. Almost 60 (90.2%) isolates of *E. coli* showed intermediate resistance to one or more antibiotics. No isolate showed complete sensitivity against all the antibiotics tested. But all the isolates showed complete sensitivity towards Chloramphenicol, Trimethoprim, Nalidixic acid, Norfloxacin, Tetracycline, Nitrofurantoin and Furazolidone (Fig. 1–4).

Table.1 Showing primers used for molecular characterization of *E. coli*

Target gene	Primer	Sequence	Product size	Annealing temperature	Reference
ermB	ermBF	GAAAAAGTACTCAACCAAATA	639	45	Nguyen <i>et al.</i> , 2009
	ermBR	AATTTAAGTACCGTTACT			
ermC	ermCF	TCAAAACATAATATAGATAAA	642	45	Nguyen <i>et al.</i> , 2009
	ermCR	GCTAATATTGTTTAAATCGTCAAT			

Table.2 Results of biochemical tests for *Escherichia coli* obtained

S. No	Tests	Positive		Negative	
		No	%	No	%
1.	Methyl red	61/67	91.67%	06/67	08.33%
2.	Voges Proskauer's	6/67	08.33%	61/67	91.67%
3.	Citrate utilization	14/67	20.83%	53/67	79.17%
4.	Indole	67/67	100.00%	-	-
5.	Nitrate reduction	67/67	100.00%	-	-
6.	ONPG	64/67	95.83%	3/67	04.17%
7.	Lysine utilization	59/67	87.50%	8/67	12.50%
8.	Lactose	64/67	95.83%	3/67	04.17%
9.	Glucose	67/67	100.00%	-	-
10.	Sucrose	64/67	95.83%	3/67	04.17%
11.	Sorbitol	50/67	75.00%	17/67	25.00%

Table.3 Antimicrobial resistant profile of isolate *E. coli*

Antibiotics	Resistant	Intermediate	Sensitive
Ampicillin	50 (67)	10(67)	7 (67)
Cephalothin	35 (67)	22 (67)	10 (67)
Aztreonam	23 (67)	15 (67)	29 (67)
Erythromycin	41 (67)	11 (67)	15 (67)
Furazolidone	-	-	67 (67)
Nalidixic acid	-	-	67 (67)
Nitrofurantoin	-	-	67 (67)
Norfloxacin	-	-	67 (67)
Trimethoprim	-	-	67 (67)
Tetracycline	-	-	67 (67)
Chloramphenicol	-	-	67 (67)
Neomycin	-	-	67 (67)

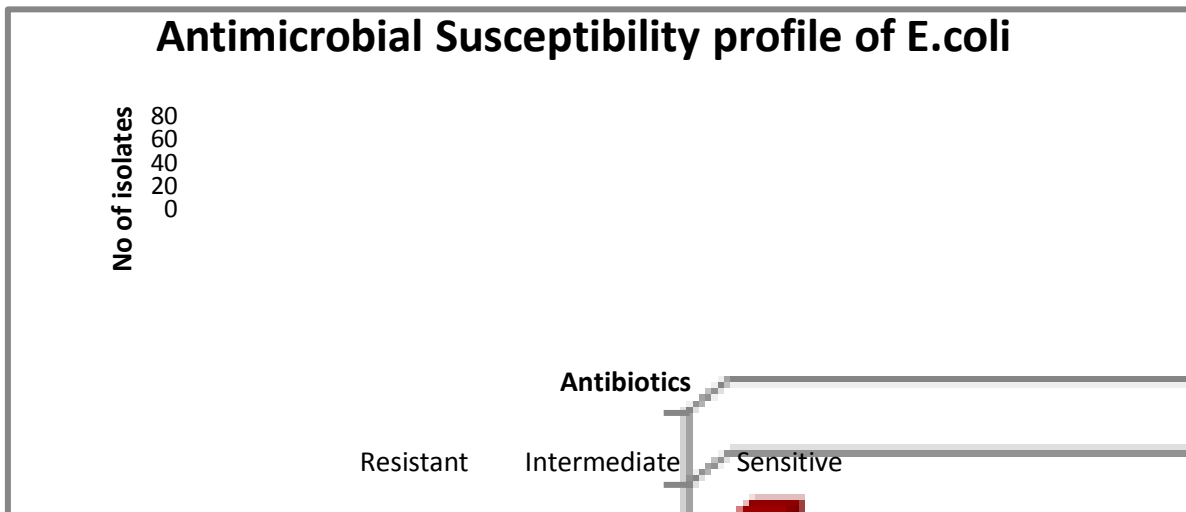


Fig.1 *Escherichia coli* on EMB agar with metallic sheen



Fig.2 Antimicrobial susceptibility test conducted on *E. coli* isolates

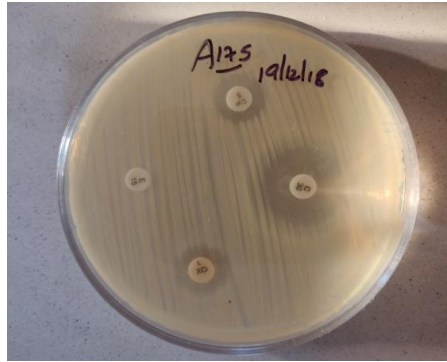


Fig.3 Samples showing positive of 639 bp *ermB* gene. Lane 1- ladder (100bp), Lane 1-5 – isolated *E.coli* samples, Lane 6- Positive control

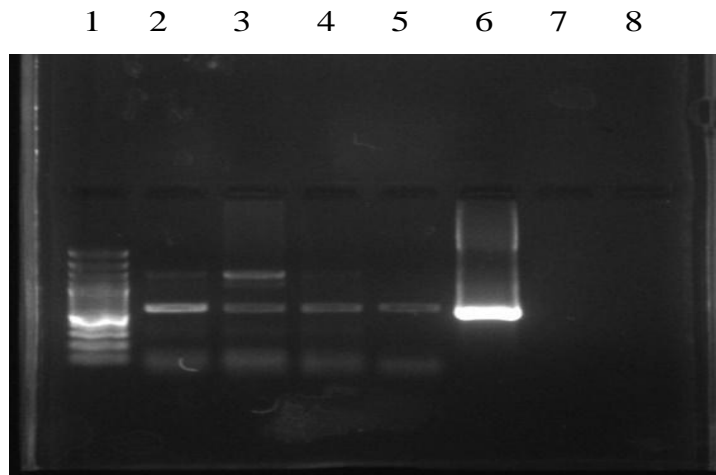
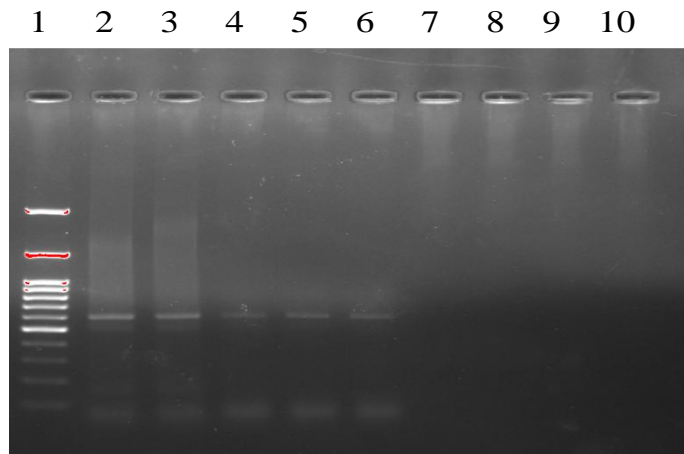


Fig.4 Samples showing positive of 642 bp *ermC* gene. Lane 1- ladder (100bp), Lane 1- Positive control, Lane 2-6 – isolated *E.coli* samples



A total of 41 isolates showed resistance against erythromycin. These bacteria were tested for the prevalence of *ermB* and *ermC* gene using primers given by Nguyen *et al.*, 2009 which yielded an amplicon of 639 bp and 642bp respectively. Erm-directed resistance can either be constitutive or macrolide inducible (Weisblum *et al.*, 1995) and it should be noted that the possession of *erm* genes was predominantly associated with resistance among *Escherichia* spp (Petersen *et al.*, 2003; Hoa *et al.*, 2008).

Of the total 41 tested, 25 bacteria were positive for *ermB* gene, which indicated a high level (61.2%) of prevalence, whereas 17 isolates showed positive for *ermC* gene, which is about 42.5% prevalence.

Due to the bioaccumulation and toxic actions of antibiotics used in farming, this emergence of the resistance genes can have an impact in environment and also in public health.

So the molecular characterization of the antimicrobial resistant genes were focused in this study to know the clear cut data on the development of antimicrobial resistance in the shrimp farming environment.

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