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Food waste as a reservoir of antibiotic resistant strains: A study on spread of ESBL linked with ABR strains from seafood waste

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Abstract
Antibiotic use and transmission including the subsequent risk of contamination resistant to antibiotics (ABR) in food ecosystems have recently come under criticism. Notably, the selective pressures exerted by residual antibiotics in the raw and processed food commodities make food waste habitats like hotel waste and food outlets waste are hotspots for the growth and transmission of antibiotic resistance genes. A major global concern is the rise of extended-spectrum beta-lactamases (ESBL) and multidrug antibiotic resistance (ABR) in bacterial populations of food waste. A total of 36 bacterial isolates were collected from food waste samples and tested for the ESBL producers and ABR resistant strains against certain standard antibiotics. The ABR profile of the bacterial isolates from seafood waste was observed against all four major antibiotics family/groups (Aminoglycosides, β-Lactams, Quinolone, and Others) as per CLSI (Clinical and Laboratory Standards Institute) standard procedure. The seafood waste isolates were highly resistant to Methicillin (5 mcg), Penicillin-G (10 mcg) and Cefotaxime (30 mcg) and about 80% of strains were resistant to at least one antibiotic. By using a combination and double disc synergy test in accordance with the recommendations of the CLSI, ESBL-producing isolates were partially identified. The results suggested the significance of resistance monitoring and related interventions by highlighting the role of food waste as a natural reservoir of bacteria that produce ABR and ESBL.

Keywords: Antibiotics resistance, seafood waste, ESBL, β-lactams, quinolone, aminoglycosides

1. Introduction
Since seafood is one of the highly perishable food commodity and processing it after the harvesting was critical task. Lack of proper harvesting and processing leads to numerous quantity of seafood waste production (Erasmus *et al*., 2021) [14]. Seafood wastage occurs at the initial stage of harvesting, at the time of processing, distribution and consumption stage. In case of fish wastage occurs during the stage of processing neatly 55% is lost, claimed to be inedible according to the FAO report (Dave C *et al*., 2015). In terms of seafood waste reduction lead to production of value-added products such as animal feed and biogas (Kafle & Kim, 2012) [15]. Increasing the Antibiotic resistance (ABR) in clinical side need to proper interventions. Antibiotic resistance in pathogenic bacteria has been a recent development, as evidenced by collections of microorganisms that precede the antibiotic era are extremely sensitive to antibiotics (D'Costa & Anil, 2011) [9]. ABR in bacteria is a global hazard to humans and livestock (Vignesh *et al*., 2016) [27] that cannot be prevented but may be managed, and it must be addressed in the most effective manner feasible (Bush *et al*., 2011). On the other hand, the problem is solely linked to the use and misuse of antibiotics in people and animals (R.I. Aminov & Mackie, 2007) [2].
Extended-spectrum of beta-lactamases (ESBL) are enzyme encrypted by the genes in the conjugative plasmids that consult resistance to a span of beta-lactam antibiotics as defined by the European Food Safety Authority panel on biological hazard (Sharifi Yazdi *et al*., 2011) [21]. The ESBL are beta-lactamases which can hydrolyze Cephalosporins and Penicillin-G (Doi *et al*., 2017) [12]. The main mechanisms for ß-lactam resistance in bacteria are the synthesis of ß-lactamases, the development of efflux pumps, changes to the outer membrane porins, and modifications to penicillin-binding proteins (Surgers *et al*., 2019) [23]. Due to the implementation and widespread use of broad-spectrum cephalosporins in pharmaceuticals in the 1980s, the first strains of *Enterobacteriaceae* that produce ESBL were identified (Castanheira *et al*., 2021) [8]. To establish opportunities to combat multidrug resistance and β-lactamases, surveillance and monitoring of food waste ecosystems is an essential step to understand the frequency of ABR & ESBL strains. To our best of our knowledge, the ABR

and ESBL patterns of bacteria from food waste samples are new approaches. The aim of the study is to understand the frequency of ABR and ESBL strains in the food waste environment.

2. Materials and Methodology

2.1 Sampling

Seafood (fish, shrimp, and crab) wastes were collected from the local market of Thanjavur city and stored in a sterile containers separately and kept in the ice box. After, reached into the lab within 3 h, the samples were blended homogeneously before the analysis. Further, the homogenized seafood sample were stored in refrigerator at 4 ºC for further analysis. In this study, single random sampling was performed and each sample was collected three times (Vignesh, Dahms, Kumarasamy, *et al*., 2015) [24].

2.2 Isolation of bacterial strains

About 1g of blended homogenized seafood sample was suspended in 10 ml of sterile ultra-Milli Q water and mixed well using a vortex for 10 min. Then, the sample were serially diluted and performed pure culture technique (spread plate technique-0.1ml sample added into the respective agar plates) (Table 1). The plates were incubated at 35 ± 1 ºC for 24-48 hours. All the trails was performed in triplicate and the mean values are presented here. The typical colonies were selected and purified by successive streaking and cultured in nutrient broth and stored in refrigerator for further analysis (Vignesh *et al*., 2014; Vignesh, Dahms, Kim, *et al*., 2015) [25, 24]. All the typical bacterial colonies from the selective media plates was observed as ("-like") to known microorganisms (LO) (Vignesh *et al*., 2014; Vignesh, Dahms, Kim, *et al*., 2015) [25, 24].

A total of 36 strains were isolated from the different types of seafood waste samples using different selective media (6 strains from each media plates). All the strains were sub cultured in nutrient broth was stored in 4℃. For the long-term storage, the culture stored in 40% glycerol stock at -20 ºC. The sterile Milli Q water was used throughout the study (Vignesh, 2021). The chemicals, reagents and microbial media used in this study were hi-grade purchased from HI-MEDIA Pvt. Ltd, Mumbai, India.

Table 1: Details of selective media used for isolation of bacterial strains

S. No.	Parameters	Cultural media	Positive colonies
1.	*Escherichia coli*	Violet red bile glucose agar (VRBG)	Pink
2.	*Vibro parahaemolyticus*	Thiosulfate citrate bile salt (TCBS)	Green
3.	*Salmonella* sp.	SS Agar	Black
4.	*Staphylococcus aureus*	Mannitol Salt Agar (MSA)	Yellow/ White
5.	*Enterococcus* sp.	M-Enterococcus Agar (MEA)	Pink - dark red
6.	*Pseudomonas* sp.	Pseudomonas isolation Agar (PIA)	Green/ white

2.3 Antibiotic resistant study (ABR)

All the bacterial strains were isolated using selective media plates i.e. 6 strains from each media plate. All the bacterial isolates were challenged against 9 Standard antibiotics on Mueller Hinton agar (MHA) for ABR studies by using Kirby-Bauer disk diffusion method (Vignesh *et al*., 2012, 2016) [28]. In this study, Cefalexin (CN), Penicillin-G (P), Norfloxacin (NX), Chloramphenicol (C), Methicillin (MET), Ciprofloxacin (CIP), Gentamicin (GEN), Cefotaxime (CTX), Streptomycin (S) antibiotics were used (Table 2). The obtained results were interpreted with CLSI standards (Clinical and Laboratory Standards Institute) and the results were classified as Resistant (the obtained result lesser than the standard CLSI value) and Sensitive (values higher than the CLSI values) (Vignesh *et al*., 2016) [27].

Table 2: Antibiotics used in the ABR study, their class, Generation and Dosage (mcg)

S. No	Antibiotic	Generation	Class	Dosage (mcg)
1.	Cefalexin-CN	First	β-lactams	30
2.	Penicillin-G P	First	β-lactams	10
3.	Norfloxacin-NX	First	Quinolone	10
4.	Chloramphenicol-C	First	Others	30
5.	Methicillin-MET	Second	β-lactams	5
6.	Ciprofloxacin-CIP	Second	Quinolone	5
7.	Gentamicin-GEN	Second	Aminoglycosides	10
8.	Cefotaxime-CTX	Third	β-lactams	30
9.	Streptomycin-S	Third	Aminoglycosides	10

2.4 Examination of ESBL positive strains

2.4.1 Primary identification of ESBL strains

All the 36 strains were challenged against the Ceftazidime (30 mcg) and Cefotaxime (30 mcg) on the MHA by the Kirby-Bauer disc diffusion method. The zone of inhibition was observed based on the CLSI guidelines for the Ceftazidime as ≤ 22 mm and Cefotaxime as ≤ 27 mm were considered as ESBL producers. Further, the strains was challenged against two antibiotics such as Amoxiclav (Augmentin-amoxicillin-clavulanic acid 20 mg/10 mg) and Ceftazidime (30 mcg) and these discs were placed at the distance of 20 mm between from the center point on the MHA. The positive ESBL strains were identified based on the augmentation of Ceftazidime expanded towards the zone of inhibition (Rajivgandhi Govindan, 2018) [18].

2.4.2 Secondary identification of ESBL producers

Among the 36 total strains, based on the Antibiotic resistant study and ESBL study reports, top 12 ABR and ESBL positive strains were further selected for the secondary identification. The selected 12 strains were challenged against Hexa 23 and Hexa 24 antibiotic discs for confirmation of ESBL producers.

2.4.3 Affirmatory test by MIC stripe method

According to CLSI guidelines, the Multi Ezy MIC strip method was performed and calculated the zone of inhibition for ESBL confirmation test. The Ezy MIC strip MIX+/MIX (MIX +0.032-4, MIX: 0.125-16) is segmented into two parts; the upper part of the strip contains Ceftazidime, Cefotaxime + Clavulanic acid mixture and the lower part of the strip contains Ceftazidime and Cefotaxime mixture (Saravanan *et al*., 2018) [19].

3. Results

The antibiotic resistant pattern was observed from the seafood waste samples underpinning the critical role of agricultural

and general communities as a source of ABR genes. The samples were collected from different locations of Thanjavur city and 6 strains from each selective media were selected for this study. Further, ESBL producing strains were explored through primary and confirmative tests.

3.1 Antibiotic resistant bacteria isolated from seafood waste

The strains were screened using the selective media and the morphology pattern (strain method) followed by biochemical characterization test. More isolates were resistant to Penicillin-G, Methicillin and Cefotaxime, whereas strains were highly susceptible to Chloramphenicol (58%), Gentamicin (83%), Streptomycin (50%) and Norfloxacin (55%). Further, the intermediate level were also observed against some of the antibiotics. The prevalence of ABR pattern in seafood waste are given in Table 3.

Table 3: Antibiotic wise resistant & sensitive strains in Seafood waste

Antimicrobials	Resistant n=36		Intermediate n=36		Sensitive n=36	
	N	%	N	%	N	%
Cefalexin-CN	11	30	4	11	20	55
Cefotaxime-CTX	36	100	0	0	0	0
Chloramphenicol-C	10	28	4	11	21	58
Ciprofloxacin-CIP	15	42	5	14	16	44
Gentamicin-GEN	5	14	1	3	30	83
Methicillin-MET	36	100	0	0	0	0
Norfloxacin-NX	6	17	8	22	20	55
Penicillin-G P	36	100	0	0	0	0
Streptomycin-S	5	14	10	28	18	50

3.2 Co-resistance of ABR isolates from seafood waste

The bacterial isolates from the seafood waste were at least resistant to 2 to 5 antimicrobials (Table 4). Especially, one bacterial strain was resistant to nearly 5 standard antibiotics. Among the seafood waste isolates, 80% of strains were resistant to Methicillin, Penicillin and Cefotaxime.

Table 4: Antimicrobial resistant, sensitive and intermediate strains in Seafood waste

Antimicrobials	Resistant n=36		Intermediate n=36		Sensitive n=36	
	N	%	N	%	N	%
1 Antimicrobials	0	0	2	5	10	28
2 Antimicrobials	0	0	6	17	8	22
3 Antimicrobials	9	25	12	33	2	5
4 Antimicrobials	13	36	7	19	0	0
5 Antimicrobials	14	38	7	19	0	0
6 Antimicrobials	0	0	2	5	0	0
7 Antimicrobials	0	0	0	0	0	0
8 Antimicrobials	0	0	0	0	0	0
9 Antimicrobials	0	0	0	0	0	0

3.3 Dispersal of ABR within the generations of antibiotics

The distribution of ABR pattern within the three generation was presented in Table 5. Among the three generation, the highest resistant was observed in the second generation (Methicillin-MET, Ciprofloxacin-CIP) (71%-51 strains) followed by first generation (Cefalexin-CN, Norfloxacin-NX, Penicillin-GP, Chloramphenicol-C) (44%-63 strains) and third generation antibiotics (42%-46 strains).

Table 5: Generation-wise Antimicrobial resistant pattern in seafood waste sample

Antibiotic-Generations	No. of strains	Resistance Details	
		N	%
First Generation Abs	144	63	44
Second generation Abs	72	51	71
Third generation Abs	108	46	42

3.4 Distribution of ABR within the antibiotic group

The Antibiotics are classified as antibiotic groups such as Aminoglycosides, β-Lactams, Quinolone and others. In the study, the highest resistant was observed in β-lactams group antibiotics followed by Aminoglycosides, Quinolone and Others (Table 6).

Table 6: Group-wise Antibiotic resistant pattern in seafood waste sample

Antibiotic-Group	No. of strains	Resistance Details	
		N	%
Aminoglycosides	72	10	14
β-Lactams	144	119	83
Quinolone	72	21	29
Others	36	10	28

3.5 ESBL production-primary identification, double disk combination test

In primary identification of ESBL study, the 12 strains were observed as positive out of 36 isolates. The test was carried out against Ceftazidime (≤ 22 mm) and Cefotaxime (≤ 27 mm) by Kirby Bauer disk diffusion method. Further, the confirmation study was carried out by the double disk combination test (DDCT) with conjunction of Ceftazidime (22 mm) and Cefotaxime (27 mm) and the results showed that the zone of antibiotic inhibition edge extended towards Augmentin.

3.6 Affirmatory test by MIC stripe method and Hexa discs diffusion

Based on primary identification and confirmation ESBL test results, 12 strains were further challenged against Hexa G minus (23 and 24) discs. Further, the strains were analyzed using MIC stripe detection method (minimum inhibitory concentration is interpreted at the intersection of the inhibition ellipse with two gradients (ceftazidime at one end) and ceftazidime plus clavulanic acid at other end). In these studies, all the 12 strains were shown positive results and confirmed as ESBL producers.

4. Discussion

This is an important study extend to our knowledge towards the role of food waste as a source for the antibiotic resistant strains and its transmittance possibilities (Vijay *et al*., 2021) [29]. The ABR related studies in food environments is minimal in the developing countries. Numerous ABR strains arise from clinical and healthcare settings and gradually shifts towards food animals (Casey Jeong *et al*., 2020) [7] and surrounding environment (Subramanya *et al*., 2021) [22]. The *Enterobacteriaceae* family has a significant number of the genetic insertional elements that could transmit ESBLs to other hosts, such as transposons, integrons and other insertional elements (Subramanya *et al*., 2021) [22]. One of the major environmental problems attributing to antibiotic pollution is the improper disposal of food waste containing antibiotic residues and resistant microorganisms in public areas (Zalewska *et al*., 2021) [30]. The present research demonstrated that the ß-lactam group antibiotics are widely used in animal husbandry and also the most prevalent ESBL genes are present in the agriculture and animal food sources (Shahin *et al*., 2021) [20]. A few studies have reported that the resistance patterns in bacteria isolated from food waste especially seafood waste, despite the essential need to examine into ABR and ESBL trends in the food environment (Durairajan *et al*., 2021) [13].

In this current research, many strains from the seafood waste were resistant to Penicillin-G (First-generation antibiotic) followed by Cefotaxime (second-generation antibiotic). This is notable finding because there are ß-lactams group antibiotics and those have the opportunity to generate cross-contamination and to transfer resistance genes over an extended period of time (Zalewska *et al*., 2021) [30]. Meanwhile, Cefalexin resistance was also dominant in isolates, it might be due to the most frequent usage of drugs in animal feed farms (Dandachi *et al*., 2018) [10]. Methicillin-resistant bacteria can be difficult to control in food exposures due biofilm formation can cause enterotoxins and affect food safety (Zehra *et al*., 2019) [31]. The presence of ABR and multidrug resistance in food waste samples denotes the extensive use of antibiotics in processed foods (Abebe, 2020) [1].

One of the prime environmental concerns relating to antibiotic contamination is the inappropriate disposal of food waste containing antibiotic residues and resistant microorganisms in public areas (Zalewska *et al*., 2021) [30]. The more number of ESBL producers in this study indicated that food waste is a crucial distributor of β-lactam-resistance to the bacterial community (R. Aminov *et al*., 2021) [3]. It includes a focus on detailed analysis methods to observe bacteria that develop biofilms in terms of managing difficulties with multidrug and β-lactamase resistance. Owing to the massive use of extended-spectrum Cephalosporins in agriculture and food animals, the majority of ESBL isolates are multi-resistant. It was observed that the ESBL-carrying bacteria which had been found on the farm using β-lactam antibiotics was also present in the pigs residing there, suggesting cross-contamination and the rapid transmission of ESBL genes (Bergšpica *et al*., 2020) [5]. The high prevalence of ESBL-positive enterobacteria identified in this study not only raises concerns about potential health implications, but it also points to seafood as a possible source of their transmission into households. Determination of ABR bacteria is an important study to understand the ABR distribution in seafood bacterial community (Asem Sanjit Singh, 2017; Dib *et al*., 2018) [4, 11]. The research reveals that seafood waste are depositories of resistant bacteria and genes that can assist the expansion of ESBL to humans. Notably, it is an important findings to understand and combat against ABR and ESBL transmittance through food waste.

5. Conclusion

This research is mainly focus on an occurrence of antibiotics resistant and ESBL producers in seafood wastes. In this study, we observed that the strains were mainly resistant to Pencillin-G, Cefotaxime and Methicillin. Especially, one bacterial strain was resistant to nearly 5 Standard antibiotics. Among the 3 generation, the highest resistant was observed in second generation antibiotics and in group-wise the Antibiotics pattern, the β-lactam group has the highest resistant. Further in this study 12 strains were shown positive results and been confirmed as ESBL producers. Hence, the detection of ABR and ESBL producers could indicate and reduce the risk of infection and increase the focus towards the health care sector.

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