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# Prevalence, Antimicrobial Resistance, and Molecular Characterization of *Escherichia coli* Isolated from Food Contact Surfaces in Seafood Pre-Processing Plants (India)

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## HIGHLIGHTS

- Enteropathogenic *Escherichia coli* strains were the most prevalent serotype.
- Shiga toxin gene *stx2* showed the highest prevalence (83.33%).
- Multi drug resistant *E. coli* strains with virulence potential showed a high risk in the seafood industry.

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***Acronyms and abbreviations***
EHEC=Enterohaemorrhagic *Escherichia coli*
ERIC=Enterobacterial Repetitive Intergenic Consensus
MAR=Multiple Antibiotic Resistance
MDR=Multi Drug Resistant
PCR=Polymerase Chain Reaction
STEC=Shiga Toxigenic *E. coli*

## ABSTRACT

**Background:** The survival of pathogens in biofilms poses a threat to food safety. The aim of this study was to determine prevalence, antimicrobial resistance, and molecular characterization of *Escherichia coli* strains.

**Methods:** Swab samples (n=144) were collected from biofilm formed on food contact surfaces in seafood pre-processing plant in India. *E. coli* was isolated and identified using *uid A* gene by Polymerase Chain Reaction (PCR). The risk assessment of the isolates was carried out in terms of their drug resistance and the presence of virulence genes. Antimicrobial susceptibility testing was performed using the Kirby-Bauer disk diffusion method. Phylogenetic grouping was done by quadruplex PCR. Molecular typing of the strains was performed by Enterobacterial Repetitive Intergenic Consensus-PCR (ERIC-PCR). Data were statistically analyzed using SPSS version 22.

**Results:** Enteropathogenic *E. coli* (EPEC) strains were the most prevalent serotype. Multiplex PCR analysis revealed the presence of shiga toxin genes (*stx1*, *stx2*), intimin (*eae*), and enterohemolysin genes (*hlyA*). Shiga toxin gene *stx2* showed the highest prevalence (83.33%). Among various phylogroups, B1 (45.56%) and B2 (30%) were the most prevalent phylogroups. Resistance to ampicillin (85.56%), piperacillin (84.44%), and cefpodoxime (85.56%) was widespread among the *E. coli* strains.

**Conclusion:** The presence of genetically heterogeneous multi drug resistant *E. coli* strains with virulence potential showed a high risk in the seafood industry.



## Introduction

At present, India is the second-largest fish producing country in the globe after China with an annual production of 14.2 million metric tonnes (Handbook on Fisheries Statistics, 2018). India has also witnessed a consider-

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able increase in aquaculture, especially the shrimp *Litopenaeus vannamei* and the genetically improved farm tilapia. Aquacultured shrimp produced in India is mostly exported to China, the USA, Japan, and countries in the European Union (EU). As per the Marine Products Exports Development Authority (MPEDA), Government of India; the country has exported 6.68 billion USD worth of seafood export, which is predominantly frozen shrimp exports (4.8 billion USD) to the USA and China.

Seafood processing, especially that of shrimp, involves processing at two independent units, such as a pre-processing facility and then a processing factory. Seafood pre-processing plants are those units that receive whole shrimp (mostly farm-raised) from different destinations, both intra-state and inter-state, where beheading and removal of shell and intestine are carried out. These are then stored in plastic crates with ice (one portion of shrimp stored with two portions of ice in a layer icing fashion) and transported in poly urethane form insulated trucks to the processing factories where value added shrimp such as individually quick frozen raw or cooked and ready to eat or breaded and battered or tempura shrimps are produced. These are then shipped to overseas markets, mostly in the USA and to the countries in the EU. The pre-processing units do not have any sophisticated machinery whereas the processing factories which produce value added shrimp are equipped with state-of-the-art machinery for various processing such as cooking, cooling, freezing, glazing, etc.

Shrimp pre-processing units evolved as a means to reduce the risk associated with cross-contamination of the finished product from the raw material in the processing factories. However, several pre-processing factories are not maintaining the standards prescribed by the Export Inspection Agency (EIA), Government of India. Poor sanitation and time-temperature abuse are common in many pre-processing centers. The pre-processing centers analyzed in the present study were maintaining relatively better sanitation and process control. The shrimps pre-processed (beheading, removal of shell, and deveining) at these centers were finally processed in higher-end processing factories to value-added shrimps such as raw and cooked, breaded and battered, tempura, etc. for export markets.

Seafood acts as a major vehicle for the transmission of several food-borne pathogens. Estuaries, coastal water bodies and the aquaculture ponds adjoining them from where the seafood is caught or raised are often contaminated by faecal pollution from the adjoining human settlements and the domestic animals they keep. Pathogenic microorganisms are often encountered in seafood harvested from such areas. Presence of *Escherichia coli* is often used to assess the faecal contamination of seafood processing plants in India and elsewhere (Kumar et al., 2005). *E. coli* is a normal inhabitant of the gut microflora of warm-blooded animals and is mostly considered as commensal. However, there are several *E. coli* strains that have acquired virulence factors and can cause diarrheal disease in healthy humans (Al-Sarawi et al., 2018; FDA, 2012). Contamination of seafood with *E. coli* from landing centers in Gujarat (Sivaraman et al., 2017), Tamil Nadu, and Cochin (Murugadas et al., 2016) as well as presence of hypervirulent strains in the Cochin estuary (Divya and Hatha, 2019) have been reported. A comprehensive study of the prevalence and risk associated with naturally occurring *E. coli* strains in the seafood industry is important because it provides information about the occurrence of pathogenic strains, their antimicrobial resistance profiles (Uyaguari-Díaz et al., 2018; Corzo-Ariyama et al., 2019), and environmental persistence through biofilm formation (Balcázar et al., 2015).

In the light of the above background, the current study was taken up to understand the prevalence of Multi Drug Resistant (MDR) *E. coli* that could form biofilms on various food contact surfaces in selected seafood pre-processing plants in Cochin. In this study, serotyping, phylotyping, and Enterobacterial Repetitive Intergenic Consensus-Polymerase Chain Reaction (ERIC-PCR) based characterization of the *E. coli* strains were carried out to understand the actual prevalence of diarrheagenic *E. coli* in the biofilms collected, and the risk associated was assessed in terms of antibiotic resistance and presence of virulence genes among them. The results of this research could help the industry to understand this hidden problem and take appropriate steps to address it as the product produced out here is intended for a global consumer.

## Materials and methods

### *Collection of samples*

Biofilm samples were collected using sterile cotton swabs monthly from two major shrimp pre-processing factories situated in Cochin (India) for two year period (March 2017-April 2019). Food contact surfaces such as processing tables where peeling and grading are done, plastic crates used to store the peeled and graded shrimp, and the rubber gloves on the worker's hands were selected for biofilm bound *E. coli* detection. Sampling was conducted just after the cleaning schedule in the pre-processing environment which involves scrubbing and washing the surface with liquid detergent followed by rinsing with 50 ppm chlorine water. A sterile stainless steel template of 25 $cm^2$ was used to swab the area from food contact surfaces. For worker's hand gloves, swabs from one full hand are used. The swabs were pressed and





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rolled over the gloves especially between the fingers. Swab samples from food contact surfaces were collected according to ISO (2004).

*Isolation and identification of E. coli*

A total of 144 swab samples were analyzed for the presence of *E. coli* according to the ISO suggested protocols for *Enterobacteriaceae*, coliforms, and *E. coli* respectively (ISO, 2006). Sterile cotton swabs dipped in sterile Lauryl Sulfate Tryptone Broth (LSTB; Hi-Media, India) were used to swab the selected food contact surfaces and worker's hand gloves. For the isolation of *E. coli*, swabs were directly inoculated into 10 ml LSTB in screw-capped bottles and transported to the laboratory in a portable ice chest. Soon after reaching the microbiology laboratory at Cochin University of Science and Technology, the tubes with samples were incubated at 37 $^{o}$C for 24 h and observed for gas production in Durham's tubes inside the medium. The tubes showing gas production were then streaked on Eosin Methylene Blue (EMB) agar (Hi-Media, India) and incubated at 37 $^{o}$C for 24 h. Typical *E. coli* like colonies with a green metallic sheen were further confirmed on Hicrome *E. coli* Agar (Hi-Media, India) by overnight incubation at 37 °C. Typical bluish-green colonies on Hicrome *E. coli* agar were picked and transferred onto nutrient agar slants for further characterization. Preliminary biochemical characterization of the presumptive isolates was done by Indole, Methyl Red, Voges-Proskauer, and Citrate (IMViC) tests. Isolates showing ++-- reaction in IMViC tests were further confirmed by PCR-based molecular detection of the specific *uidA* gene (Antony et al., 2016). The Genomic DNA of the isolates was extracted by the boiling method (Antony et al., 2016). Primers used in this study were summarized in Table 1. Confirmed *E. coli* strains were serotyped at National *Salmonella* and *Escherichia* Centre, Central Research Institute, Kasauli, Himachal Pradesh, India.

*Phylogenetic grouping and genotyping of isolates*

Phylogenetic groups of the strains were determined by quadruplex PCR as previously described (Clermont et al., 2013). Molecular genotyping of antibiotic resistant *E. coli* isolates was done using ERIC-PCR with primer ERIC-2 (Divya and Hatha, 2019).

*Detection of toxigenic/virulence genes in E. coli by multiplex PCR*

The presence of shiga toxin genes (*stx1* and *stx2*) and virulence genes such as intimin gene (*eae*) and enterohemolysin gene (*hlyA*) was detected using multiplex PCR as described by Paton and Paton (1998).

*Antibiotic susceptibility test*

Antibiotic susceptibility testing of the 90 *E. coli* isolates was performed using the agar disk diffusion method (Bauer et al., 1966), following the Clinical and Laboratory Standards Institute (CLSI, 2016) guidelines. The Multiple Antibiotic Resistance (MAR) Index of isolates was determined by the calculating the ratio of the number of antibiotics to which the isolate showed resistance to the total number of antibiotics to which the isolate was exposed. Isolates that are resistant to more than three antibiotics were considered as multiple antibiotic-resistant and the resistance patterns were worked out (Osundiya et al., 2013).

*Statistical analysis*

Statistical analysis was performed using IBM SPSS version 22 (IBM Corporation, United States). *P*-value<0.05 was considered statistically significant. Pearson's Chi-square test was used to test whether phylogroups, virulent genes, and antibiotic resistance were evenly distributed among different food contact surfaces.

## Results

*Prevalence of E. coli serotypes in biofilms*

In the present study, a qualitative analysis of 144 swab samples from biofilm formed on food contact surfaces in seafood pre-processing plant was performed for the presence of *E. coli.* The prevalence of various serotypes of *E. coli* in the biofilm samples from different food contact surfaces is given in Table 2.

Eighty-eight swabs (61.11%) were positive for *E. coli*. A total of 110 presumptive *E. coli* that appeared as green metallic sheen colonies on EMB agar plate were picked. Of the 110 isolates, 90 of them produced bluish-green coloured colonies (specific for *E. coli*) on Hichrome *E. coli* agar. All 90 isolates were confirmed as *E. coli* by PCR assay targeting the species-specific *uid A* gene (147 bp; Figure 1).

Maximum isolation of *E. coli* strains was made from processing tables made of stainless steel (83.3%) when compared to plastic crates (58.33%) and the rubber gloves on worker's hands (41.67%). Serotyping of the *E. coli* isolates revealed the presence of 20 different serotypes with varying degrees of prevalence (Table 3). Enteropathogenic *E. coli* (EPEC) contributed nearly 40%, followed by uropathogenic *E. coli* (UPEC; 24.4%), enterotoxigenic *E. coli* (ETEC; 14.4%), and shiga toxigenic *E. coli* (STEC) or enterohaemorrhagic *E. coli* (EHEC; 13.3%).



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*Distribution of E. coli phylogroups and genotyping*

Phylogrouping revealed the presence of five different phylogroups namely A, B1, B2, C, and E from food contact surfaces in seafood pre-processing plants. The commensal phylogroup, B1 (44.4%) is the most prevalent one, which is closely followed by the pathogenic phylogroup, B2 (31.1%).

Among 90 *E. coli* isolates, 81 were selected for molecular genotyping based on the drug resistance property. The results revealed wide genetic diversity among the strains isolated (Figure 2). The fingerprints obtained from the ERIC-PCR typing of 81 *E. coli* isolates showed a DNA banding profile consisting of 1-7 amplified bands with sizes ranging from 150 bp to 1200 bp. There were eight clusters of *E. coli* isolates from food contact surfaces, suggesting a single clone contaminant.

*Occurrence of virulence genes in E. coli*

Multiplex PCR analysis of the 90 *E. coli* strains revealed the presence of shiga toxin genes (*stx1*, *stx2*), intimin (*eae*) gene, and enterohemolysin gene (*hlyA)* among these strains. Amplicons having product sizes of 180, 255, 384, and 534 bp indicated the presence of *stx1*, *stx2*, *eae*, and *hlyA*, respectively (Figure 3).

In the present study, a total of 83 strains (92.22%) showed the presence of one or more virulence genes (Table 4). The virulence genes such as *stx1*, *stx2*, *eae*, and *hlyA* were found to co-exist in 30% of the *E. coli* isolates. Variations in the prevalence of virulence genes among isolates from different food contact surfaces was found to be statistically significant ($p<0.05$). In the present study, the most frequently occurred virulent gene combination was *stx1/stx2/eae/hlyA* (30%).

Among the various *E. coli* phylogroups, the phylogroup E had more virulent genes than isolates belonging to other phylogroups. As far as the distribution of virulence genes among the different phylogenetic groups concerned, the most prevalent genes in group B1, B2, and E isolates were *stx1+stx2+eae+hly* combination. In general, *stx2* was the most prevalent virulent gene among various phylogroups of *E. coli*, which ranged from 66.67% in group A to 100% in groups E and C.

*Antibiotic susceptibility test*

Prevalence of antibiotic resistance was relatively high against ampicillin and cefpodoxime (85.56%), followed by piperacillin (84.44%), tigecycline (42.2%), and nitrofurantoin (36.7%). However, all the strains were susceptible to chloramphenicol, ceftriaxone, cefoxitin, and gentamycin (Figure 4).

Strains from the various food contact surfaces showed significant differences in the prevalence of antibiotic resistance except that towards piperacillin-tazobactam, gentamicin, ceftriaxone, cefoxitin, nitrofurantoin, ciprofloxacin, nalidixic acid, chloramphenicol, and trimethoprim ($p<0.05$; Table 5). Isolates from processing table and plastic crates showed significantly higher resistance ($p<0.05$) towards ampicillin, piperacillin, meropenem, and co-trimoxazole than from worker's hands.

MAR indices of *E. coli* strains and the antibiotic resistance patterns is shown in Table 6. The MAR indices ranged from 0.14-0.41. Nearly 65% of the *E. coli* isolates had an index ≥0.2. A total of 37 different antibiotic resistance patterns were observed. The most commonly observed resistance pattern among *E. coli* isolates from all three surfaces was Amp/Pi/Cpd.

Antibiotic-resistant *E. coli* isolates mainly belonged to phylogenetic groups B1, B2, E, and C. Phylogenetic group B1 showed significantly higher ($p<0.05$) antibiotic resistance than isolates belonging to other phylogenetic groups (Figure 5). All phylogenetic groups B2, E, and C strains from stainless steel surfaces were resistant to antibiotics. None of the isolates belonging to phylogroup A was resistant to any antibiotics.

**Table 1:** Primers used in the study

| Genes investigated | Primer sequence (5'-3') | | Annealing temperature | References |
|---|---|---|---|---|
| | Forward | Reverse | | |
| **Phylogenetic grouping genes** | | | | |
| *chuA* (288 bp) | atggtaccggacgaaccaac | tgccgccagtaccaaagaca | 59 °C | Clermont et al. (2013) |
| *yjaA* (211 bp) | caaacgtgaagtgtcaggag | aatgcgttcctcaacctgtg | 59 °C | Clermont et al. (2013) |
| *tspE4C2* (152 bp) | cactattcgtaaggtcatcc | agtttatcgctgcgggtcgc | 59 °C | Clermont et al. (2013) |
| *arpA* (400 bp) | aacgctattcgccagcttgc | tctccccataccgtacgcta | 59 °C | Clermont et al. (2013) |
| **Virulent genes** | | | | |
| *stx1* (180 bp) | ataaatcgccattc | agaacgcccactg | 58 °C | Paton and Paton (1998) |
| *stx2* (255 bp) | ggcactgtctgaaa | tcgccagttatctg | 58 °C | Paton and Paton (1998) |
| *eae* (384 bp) | gacccggcacaag | ccacctgcagcaac | 58 °C | Paton and Paton (1998) |
| *hlyA* (534 bp) | gcatcatcaagcgt | aatgagccaagct | 58 °C | Paton and Paton (1998) |



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**Table 2:** Prevalence and diversity of *Escherichia coli* serotypes in biofilms formed on various food contact surfaces

| Food contact surface | No. of samples | Prevalence of *E. coli* (%) | *E. coli* serotypes |
|---|---|---|---|
| Processing tables | 48 | 83.33 | O149, O22, O88, O17, O83, O128, O11, O119, O63, O126, O129, O2, O35, O49, O157, O7, O118 |
| Plastic crates | 48 | 58.33 | O149, O22, O88, O17, O83, O119, O63, O35, O118, O166 |
| Worker's hand gloves | 48 | 41.67 | O149, O22, O88, O83, O119, O10, O117 |

**Table 3:** Overall and relative prevalence of various serotypes of *Escherichia coli* in biofilms on food contact surfaces

| *Escherichia coli* serotype | Overall prevalence (%) | Prevalence on various food contact surfaces (%) | | |
|---|---|---|---|---|
| | | Stainless steel | Plastic | Rubber |
| O88 [a] | 18.89 | 16.13 | 14.29 | 35.71 |
| O22 [b] | 14.44 | 16.13 | 7.14 | 14.29 |
| O149 [c] | 10.00 | 9.68 | 7.14 | 14.29 |
| O83 [b] | 7.78 | 6.45 | 7.14 | 14.29 |
| O17 [d] | 6.67 | 8.06 | 7.14 | 0 |
| O119 [a] | 6.67 | 3.23 | 21.43 | 7.14 |
| O126 [a] | 6.67 | 9.68 | 0 | 0 |
| O11 [c] | 3.33 | 4.84 | 0 | 0 |
| O63 [a] | 3.33 | 3.23 | 7.14 | 0 |
| O118 [d] | 2.22 | 1.61 | 7.14 | 0 |
| O35 [d] | 2.22 | 1.61 | 7.14 | 0 |
| O166 [a] | 1.11 | 0 | 7.14 | 0 |
| O10 [a] | 1.11 | 0 | 0 | 7.14 |
| O128 [a] | 1.11 | 1.61 | 0 | 0 |
| O129 [a] | 1.11 | 1.61 | 0 | 0 |
| O2 [b] | 1.11 | 1.61 | 0 | 0 |
| O49 [c] | 1.11 | 1.61 | 0 | 0 |
| O157 [d] | 1.11 | 1.61 | 0 | 0 |
| O7 [b] | 1.11 | 1.61 | 0 | 0 |
| O117 [d] | 1.11 | 0 | 0 | 7.14 |
| UT [e] | 5.56 | 8.06 | 0 | 0 |

[a] Enteropathogenic *E. coli* (EPEC), [b] Uropathogenic *E. coli* (UPEC), [c] Enterotoxigenic *E. coli* (ETEC), [d] Shiga toxigenic *E. coli* (STEC) or Enterohaemorrhagic *E. coli* (EHEC),
[e] Untypeable (UT)

**Table 4:** Distribution of virulence genes among *Escherichia coli* from various food contact surfaces

| Virulence genes | Overall percentage of prevalence (n=90) | Prevalence (%) among *Escherichia coli* from | | |
|---|---|---|---|---|
| | | Processing tables (n=62) | Plastic crates (n=14) | Rubber gloves (n=14) |
| *stx1* | 0 | 0 | 0 | 0 |
| *stx2* | 20 | 17.74 | 28.57 | 21.42 |
| *eae* | 1.11 | 1.61 | 0 | 0 |
| *hly* [a,b] | 1.11 | 0 | 7.14 | 0 |
| *stx1+stx2* [b] | 6.67 | 6.45 | 0 | 14.29 |
| *eae+hly* [b,c] | 6.67 | 8.06 | 7.14 | 0 |
| *stx2+eae+hly* [b,c] | 6.67 | 8.06 | 7.14 | 0 |
| *stx2+hly* [a,b] | 10 | 11.29 | 0 | 14.29 |
| *stx1+stx2+eae* | 1.11 | 1.61 | 0 | 0 |
| *stx1+stx2+hly* [a,b] | 8.89 | 6.45 | 21.42 | 7.14 |
| *stx1+stx2+eae+hly* | 30 | 29.03 | 35.71 | 28.57 |

Statistically significant differences ($p<0.05$) in the prevalence of virulent genes in *E. coli* between food contact surfaces are indicated by superscript letters; [a] between steel and plastic, [b] between plastic and rubber, [c] between steel and rubber



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**Table 5:** Prevalence of antibiotic resistance in *Escherichia coli* isolated from food contact surfaces in seafood processing plants

| Antibiotics | Antibiotic resistance (%) | | | |
|---|---|---|---|---|
| | Processing tables (n=62) | Plastic crates (n=14) | Rubber gloves (n=14) | Total (n=90) |
| Ampicillin (10 µg) [b,c] | 88.70 | 85.71 | 71.42 | 85.56 |
| Piperacillin (100 µg) [a,c] | 88.70 | 13.33 | 10.00 | 84.44 |
| Ampicillin-Sulbactam (10/10 µg) [b,c] | 0 | 0 | 7.14 | 1.11 |
| Piperacillin-Tazobactam (100/10 µg) | 1.61 | 0 | 0 | 1.11 |
| Gentamicin (10 µg) | 0 | 0 | 0 | 0 |
| Streptomycin (10 µg) [a] | 19.35 | 14.29 | 21.43 | 18.89 |
| Colistin (10 µg) [a,c] | 8.06 | 28.57 | 21.43 | 13.33 |
| Imipenem (10 µg) [a,b] | 19.35 | 42.86 | 14.29 | 22.22 |
| Meropenem (10 µg) [b,c] | 12.90 | 7.14 | 0 | 10.00 |
| Cefpodoxime (30 µg) [b,c] | 88.7 | 85.71 | 71.43 | 85.56 |
| Ceftazidime (30 µg) [b,c] | 17.74 | 21.43 | 35.71 | 21.11 |
| Ceftriaxone (30 µg) | 0 | 0 | 0 | 0 |
| Cefotaxime (30 µg) [b,c] | 14.51 | 14.29 | 28.57 | 16.67 |
| Cefoxitin (30 µg) | 0 | 0 | 0 | 0 |
| Nitrofurantoin (30 µg) | 35.48 | 42.86 | 35.71 | 36.67 |
| Ciprofloxacin (5 µg) | 1.61 | 0 | 0 | 1.11 |
| Nalidixic Acid (30 µg) | 8.06 | 7.14 | 14.29 | 8.89 |
| Co-Trimoxazole (25 µg) [b,c] | 4.84 | 7.14 | 0 | 4.44 |
| Tetracycline (30 µg) [b,c] | 8.06 | 14.29 | 0 | 7.78 |
| Chloramphenicol (30 µg) | 0 | 0 | 0 | 0 |
| Trimethoprim (5 µg) | 4.83 | 7.14 | 7.14 | 5.56 |
| Tigecycline (15 µg) [b,c] | 46.77 | 42.86 | 21.42 | 42.22 |

**Note:** Statistically significant differences ($p$<0.05) in the prevalence of antibiotic resistance between stations are indicated by superscript letters; [a] between steel and plastic, [b] between steel and rubber, [c] between plastic and rubber

**Table 6:** Multiple Antibiotic Resistance (MAR) index and antibiotic resistance patterns of *Escherichia coli* strains

| MAR index | Antibiotic resistance pattern | No. of isolates (%) |
|---|---|---|
| 0.14 | Amp, Pi, Cpd | 19 (21.1) |
| 0.18 | Amp, Pi, Cpd, Tgc<br>Amp, Pi, Cpd, Na<br>Amp, Pi, Cpd, Na, Te | 7 (7.8) |
| 0.23 | Amp, Pi, Ipm, Cpd, Nit<br>Amp, Pi, Cl, Cpd, Caz<br>Amp, Pi, Pit, Cpd, Tgc<br>Amp, Pi, Cpd, Nit, Tgc<br>Amp, Pi, Mrp, Cpd, Na, Te<br>Amp, Pi, Ipm, Cpd, Caz, Ctx<br>Amp, Pi, Cl, Ipm, Cpd, Nit<br>Amp, Cl, Ipm, Cpd, Nit, Na<br>Amp, Pi, Cl, Ipm, Cpd, Tgc | 8 (8.9) |
| 0.27 | Amp, Pi, Cl, Ipm, Cpd, Nit<br>Amp, Pi, A/S, Cpd, Na, Tr<br>Amp, Pi, S, Cpd, Nit, Tgc<br>Amp, Pi, Cpd, Caz, Nit, Tgc<br>Amp, Pi, Ipm, Cpd, Ctx, Tgc<br>Amp, Pi, S, Cpd, Caz, Ctx<br>Amp, Pi, S, Cpd, Caz, Tgc<br>Amp, Pi, Cpd, Caz, Nit, Tgc<br>Amp, Pi, Cpd, Nit, Cot, Te, Tr<br>Amp, Pi, Ipm, Cpd, Cot, Te, Tr<br>Amp, Pi, Cl, Ipm, Mrp, Cpd, Nit | 21 (23.3) |
| 0.32 | Amp, Pi, Cl, Ipm, Cpd, Nit, Te<br>Amp, Pi, Cpd, Caz, Ctx, Nit, Tgc<br>Amp, Pi, S, Cpd, Ctx, Nit, Tgc<br>Amp, Pi, Ipm, Mrp, Cpd, Ctx, Tgc<br>Amp, Pi, Ipm, Mrp, Cpd, Na, Tgc<br>Amp, Pi, S, Cpd, Caz, Nit, Tgc | 11 (12.2) |
| 0.36 | Amp, Pi, S, Cpd, Caz, Ctx, Nit, Tgc<br>Amp, Pi, S, Ipm, Cpd, Caz, Nit, Tgc<br>Amp, Pi, Ipm, Cpd, Caz, Ctx, Nit, Tgc | 7 (7.8) |
| 0.41 | Amp, Pi, Cl, Ipm, Mrp, Cpd, Nit, Cot, Tr<br>Amp, Pi, Mrp, Cpd, Cip, Na, Cot, Te, Tr<br>Amp, Pi, S, Cl, Mrp, Cpd, Ctx, Nit, Tgc<br>Amp, Pi, S, Ipm, Cpd, Caz, Ctx, Nit, Tgc | 4 (4.4) |

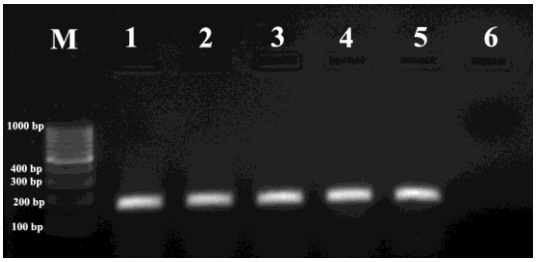


**Figure 1:** Polymerase Chain Reaction (PCR) amplified *Escherichia coli* specific *uid A* gene. Lane M: 100 bp molecular weight ladder; lanes 1-4: *uid A* gene of *E. coli* isolates; lane 5: positive control (*E. coli*- Genbank Accession No. EF2V4); lane 6: negative control



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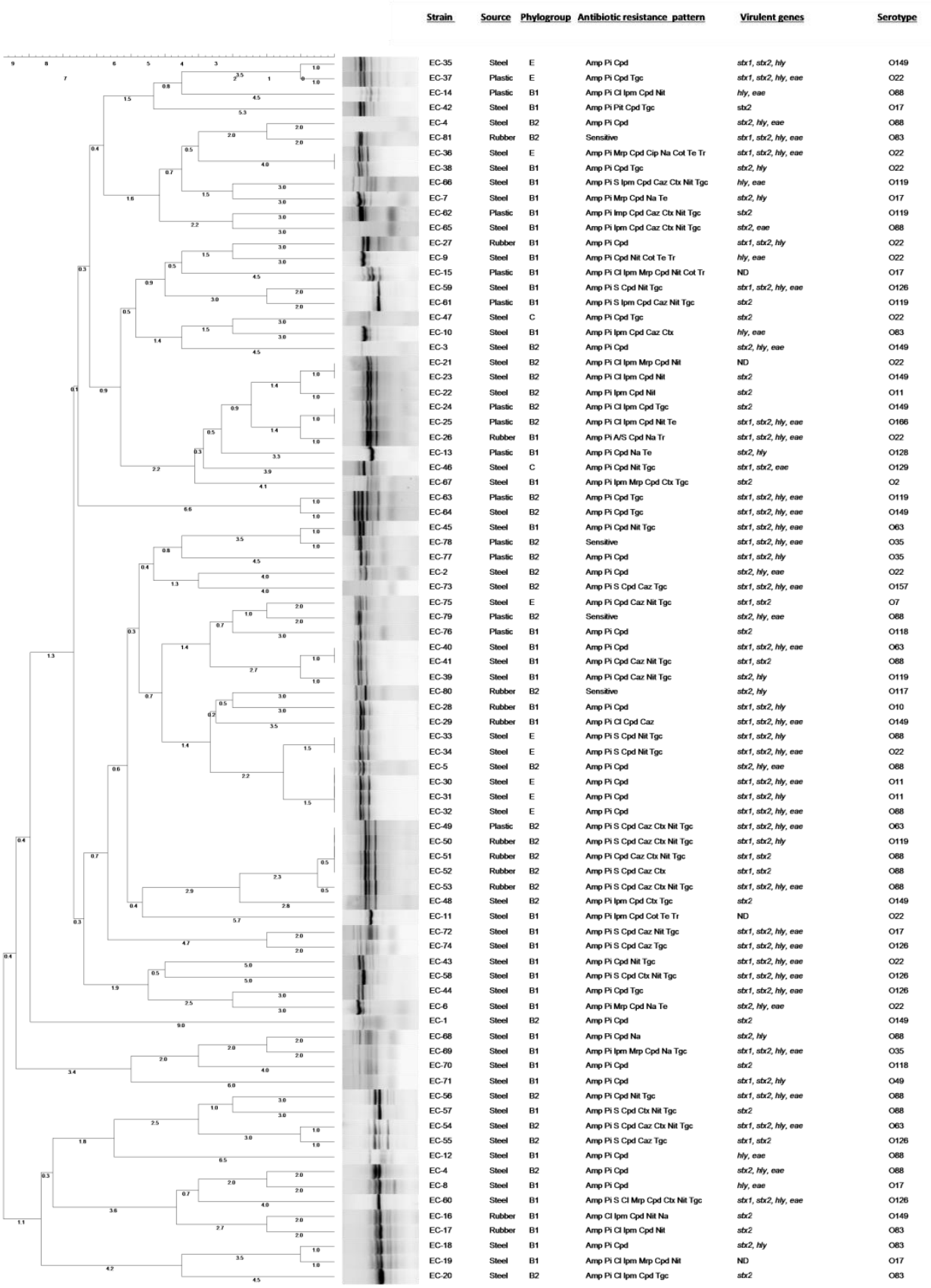

| Strain | Source | Phylogroup | Antibiotic resistance pattern | Virulent genes | Serotype |
|---|---|---|---|---|---|
| EC-35 | Steel | E | Amp Pi Cpd | *stx1, stx2, hly* | O149 |
| EC-37 | Plastic | E | Amp Pi Cpd Tgc | *stx1, stx2, hly, eae* | O22 |
| EC-14 | Plastic | B1 | Amp Pi Cl Ipm Cpd Nit | *hly, eae* | O88 |
| EC-42 | Steel | B1 | Amp Pi Pit Cpd Tgc | *stx2* | O17 |
| EC-4 | Steel | B2 | Amp Pi Cpd | *stx2, hly, eae* | O88 |
| EC-81 | Rubber | B2 | Sensitive | *stx1, stx2, hly, eae* | O83 |
| EC-36 | Steel | E | Amp Pi Mrp Cpd Cip Na Cot Te Tr | *stx1, stx2, hly, eae* | O22 |
| EC-38 | Steel | B1 | Amp Pi Cpd Tgc | *stx2, hly* | O22 |
| EC-66 | Steel | B1 | Amp Pi S Ipm Cpd Caz Ctx Nit Tgc | *hly, eae* | O119 |
| EC-7 | Steel | B1 | Amp Pi Mrp Cpd Na Te | *stx2, hly* | O17 |
| EC-62 | Plastic | B1 | Amp Pi Imp Cpd Caz Ctx Nit Tgc | *stx2* | O119 |
| EC-65 | Steel | B1 | Amp Pi Ipm Cpd Caz Ctx Nit Tgc | *stx2, eae* | O88 |
| EC-27 | Rubber | B1 | Amp Pi Cpd | *stx1, stx2, hly* | O22 |
| EC-9 | Steel | B1 | Amp Pi Cpd Nit Cot Te Tr | *hly, eae* | O22 |
| EC-15 | Plastic | B1 | Amp Pi Cl Ipm Mrp Cpd Nit Cot Tr | ND | O17 |
| EC-59 | Steel | B1 | Amp Pi S Cpd Nit Tgc | *stx1, stx2, hly, eae* | O126 |
| EC-61 | Plastic | B1 | Amp Pi S Ipm Cpd Caz Nit Tgc | *stx2* | O119 |
| EC-47 | Steel | C | Amp Pi Cpd Tgc | *stx2* | O22 |
| EC-10 | Steel | B1 | Amp Pi Ipm Cpd Caz Ctx | *hly, eae* | O83 |
| EC-3 | Steel | B2 | Amp Pi Cpd | *stx2, hly, eae* | O149 |
| EC-21 | Steel | B2 | Amp Pi Cl Ipm Mrp Cpd Nit | ND | O22 |
| EC-23 | Steel | B2 | Amp Pi Cl Ipm Cpd Nit | *stx2* | O149 |
| EC-22 | Steel | B2 | Amp Pi Ipm Cpd Nit | *stx2* | O11 |
| EC-24 | Plastic | B2 | Amp Pi Cl Ipm Cpd Tgc | *stx2* | O149 |
| EC-25 | Plastic | B2 | Amp Pi Cl Ipm Cpd Nit Te | *stx1, stx2, hly, eae* | O166 |
| EC-26 | Rubber | B1 | Amp Pi A/S Cpd Na Tr | *stx1, stx2, hly, eae* | O22 |
| EC-13 | Plastic | B1 | Amp Pi Cpd Na Te | *stx2, hly* | O128 |
| EC-46 | Steel | C | Amp Pi Cpd Nit Tgc | *stx1, stx2, eae* | O129 |
| EC-67 | Steel | B1 | Amp Pi Ipm Mrp Cpd Ctx Tgc | *stx2* | O2 |
| EC-63 | Plastic | B2 | Amp Pi Cpd Tgc | *stx1, stx2, hly, eae* | O119 |
| EC-64 | Steel | B2 | Amp Pi Cpd Tgc | *stx1, stx2, hly, eae* | O149 |
| EC-45 | Steel | B1 | Amp Pi Cpd Nit Tgc | *stx1, stx2, hly, eae* | O63 |
| EC-78 | Plastic | B2 | Sensitive | *stx1, stx2, hly, eae* | O35 |
| EC-77 | Plastic | B2 | Amp Pi Cpd | *stx1, stx2, hly* | O35 |
| EC-2 | Steel | B2 | Amp Pi Cpd | *stx2, hly, eae* | O22 |
| EC-73 | Steel | B2 | Amp Pi S Cpd Caz Tgc | *stx1, stx2, hly, eae* | O157 |
| EC-75 | Steel | E | Amp Pi Cpd Caz Nit Tgc | *stx1, stx2* | O7 |
| EC-79 | Plastic | B2 | Sensitive | *stx2, hly, eae* | O88 |
| EC-76 | Plastic | B1 | Amp Pi Cpd | *stx2* | O118 |
| EC-40 | Steel | B1 | Amp Pi Cpd | *stx1, stx2, hly, eae* | O63 |
| EC-41 | Steel | B1 | Amp Pi Cpd Caz Nit Tgc | *stx1, stx2* | O88 |
| EC-39 | Steel | B1 | Amp Pi Cpd Caz Nit Tgc | *stx2, hly* | O119 |
| EC-80 | Rubber | B2 | Sensitive | *stx2, hly* | O117 |
| EC-28 | Rubber | B1 | Amp Pi Cpd | *stx1, stx2, hly* | O10 |
| EC-29 | Rubber | B1 | Amp Pi Cl Cpd Caz | *stx1, stx2, hly, eae* | O149 |
| EC-33 | Steel | E | Amp Pi S Cpd Nit Tgc | *stx1, stx2, hly* | O88 |
| EC-34 | Steel | E | Amp Pi S Cpd Nit Tgc | *stx1, stx2, hly, eae* | O22 |
| EC-5 | Steel | B2 | Amp Pi Cpd | *stx2, hly, eae* | O88 |
| EC-30 | Steel | E | Amp Pi Cpd | *stx1, stx2, hly, eae* | O11 |
| EC-31 | Steel | E | Amp Pi Cpd | *stx1, stx2, hly* | O11 |
| EC-32 | Steel | E | Amp Pi Cpd | *stx1, stx2, hly, eae* | O88 |
| EC-49 | Plastic | B2 | Amp Pi S Cpd Caz Ctx Nit Tgc | *stx1, stx2, hly, eae* | O63 |
| EC-50 | Rubber | B2 | Amp Pi S Cpd Caz Ctx Nit Tgc | *stx1, stx2, hly* | O119 |
| EC-51 | Rubber | B2 | Amp Pi Cpd Caz Ctx Nit Tgc | *stx1, stx2* | O88 |
| EC-52 | Rubber | B2 | Amp Pi S Cpd Caz Ctx | *stx1, stx2* | O88 |
| EC-53 | Rubber | B2 | Amp Pi S Cpd Caz Ctx Nit Tgc | *stx1, stx2, hly, eae* | O88 |
| EC-48 | Steel | B2 | Amp Pi Ipm Cpd Ctx Tgc | *stx2* | O149 |
| EC-11 | Steel | B1 | Amp Pi Ipm Cpd Cot Te Tr | ND | O22 |
| EC-72 | Steel | B1 | Amp Pi S Cpd Caz Nit Tgc | *stx1, stx2, hly, eae* | O17 |
| EC-74 | Steel | B1 | Amp Pi S Cpd Caz Tgc | *stx1, stx2, hly, eae* | O126 |
| EC-43 | Steel | B1 | Amp Pi Cpd Nit Tgc | *stx1, stx2, hly, eae* | O22 |
| EC-58 | Steel | B1 | Amp Pi S Cpd Ctx Nit Tgc | *stx1, stx2, hly, eae* | O126 |
| EC-44 | Steel | B1 | Amp Pi Cpd Tgc | *stx1, stx2, hly, eae* | O126 |
| EC-6 | Steel | B1 | Amp Pi Mrp Cpd Na Te | *stx2, hly, eae* | O22 |
| EC-1 | Steel | B2 | Amp Pi Cpd | *stx2* | O149 |
| EC-68 | Steel | B1 | Amp Pi Cpd Na | *stx2, hly* | O88 |
| EC-69 | Steel | B1 | Amp Pi Ipm Mrp Cpd Na Tgc | *stx1, stx2, hly, eae* | O35 |
| EC-70 | Steel | B1 | Amp Pi Cpd | *stx2* | O118 |
| EC-71 | Steel | B1 | Amp Pi Cpd | *stx1, stx2, hly* | O49 |
| EC-56 | Steel | B2 | Amp Pi Cpd Nit Tgc | *stx1, stx2, hly, eae* | O88 |
| EC-57 | Steel | B1 | Amp Pi S Cpd Ctx Nit Tgc | *stx2* | O88 |
| EC-54 | Steel | B2 | Amp Pi S Cpd Caz Ctx Nit Tgc | *stx1, stx2, hly, eae* | O63 |
| EC-55 | Steel | B2 | Amp Pi S Cpd Caz Tgc | *stx1, stx2* | O126 |
| EC-12 | Steel | B1 | Amp Pi Cpd | *hly, eae* | O88 |
| EC-4 | Steel | B2 | Amp Pi Cpd | *stx2, hly, eae* | O88 |
| EC-8 | Steel | B1 | Amp Pi Cpd | *hly, eae* | O17 |
| EC-60 | Steel | B1 | Amp Pi S Cl Mrp Cpd Ctx Nit Tgc | *stx1, stx2, hly, eae* | O126 |
| EC-16 | Rubber | B1 | Amp Cl Ipm Cpd Nit Na | *stx2* | O149 |
| EC-17 | Rubber | B1 | Amp Pi Cl Ipm Cpd Nit | *stx2* | O83 |
| EC-18 | Steel | B1 | Amp Pi Cpd | *stx2, hly* | O83 |
| EC-19 | Steel | B1 | Amp Pi Cl Ipm Mrp Cpd Nit | ND | O17 |
| EC-20 | Steel | B2 | Amp Pi Cl Ipm Cpd Tgc | *stx2* | O83 |

**Figure 2:** Genetic heterogeneity among antibiotic-resistant *Escherichia coli* isolated from food contact surfaces revealed by Enterobacterial Repetitive Intergenic Consensus-Polymerase Chain Reaction (ERIC-PCR)



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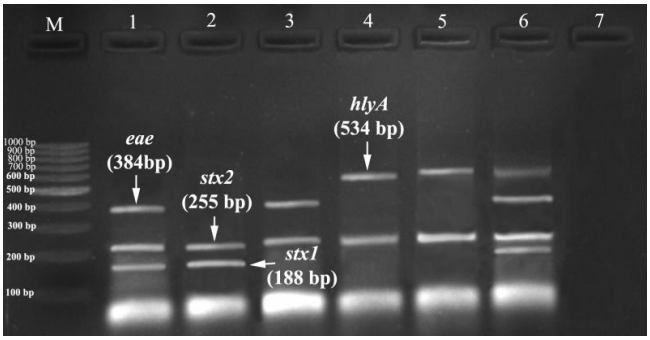


**Figure 3:** Gel image of Polymerase Chain Reaction (PCR) products after agarose gel electrophoresis with representative isolates carrying various toxigenic/virulence genes in *Escherichia coli*. Lane M: 100 bp ladder; lane 1: *stx1*, *stx2*, and *eae* genes; lanes 2: *stx1* and *stx2* genes; lane 3: *stx2* and *eae* genes; lane 4 and lanes5: *stx2*, and *hlyA* genes; lanes 6: Positive Control (*E. coli* - Genbank Accession No. EF2V4): *stx1*, *stx2*, *eae*, and *hlyA* genes; lane 7: Negative control

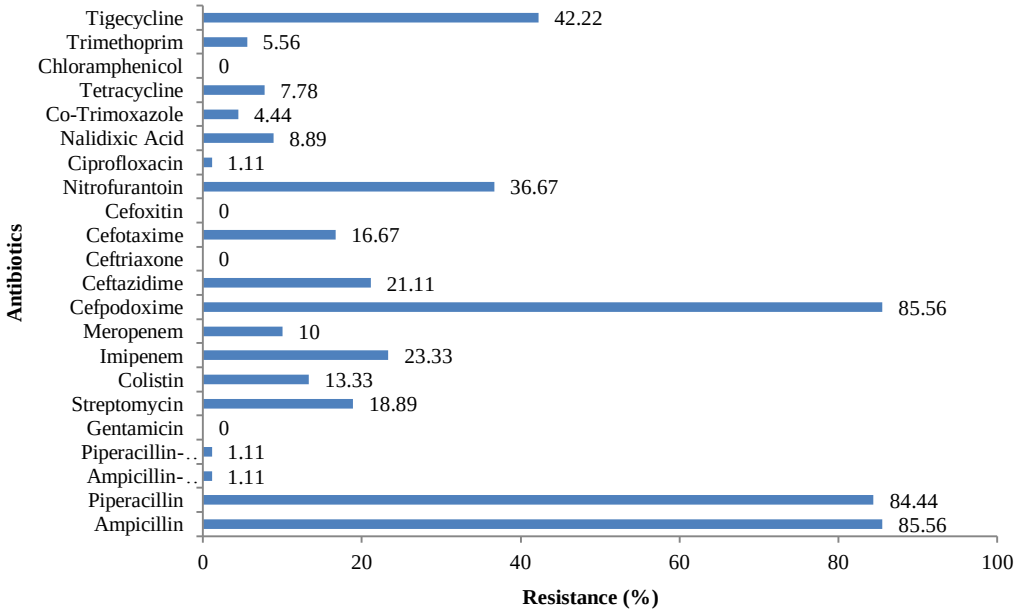


**Figure 4:** Percentage of antibiotic resistance among *Escherichia coli* from food contact surfaces in seafood processing plants (n=90)

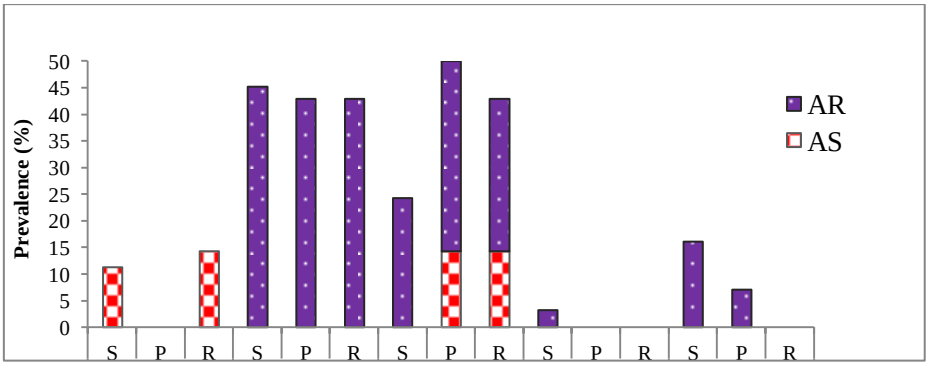


**Figure 5:** Phylogenetic group distribution among *Escherichia coli* isolates from food contact surfaces in the seafood processing plant. AR, antibiotic-resistant; AS, antibiotic sensitive; S, steel; P, plastic; R, rubber





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## Discussion

Biofilm formation on food contact surfaces and the prevalence of virulent and MDR *E. coli* in it poses food safety issues in the seafood pre-processing plants. Even though the prevalence of *E. coli* in seafood from India has been previously reported (Dutta and Sengupta, 2016; Gupta et al., 2013), to the best of our knowledge, the present study is the first report of the isolation and risk assessment of the *E. coli* from biofilms associated with food contact surfaces in seafood pre-processing centers. *E. coli* strains entering the food processing environment either through contaminated food or through carriers handling seafood may survive on the premises by forming a biofilm on various surfaces. It has been suggested that surface hydrophobicity may influence the distribution and the attachment of bacteria on surfaces (Park and Kang, 2017). Due to hydrophobic interactions between food contact surfaces and bacteria, it can often form biofilms on hydrophilic surfaces such as stainless steel. Previous studies have revealed that worker's hands often harbor fecal coliforms and *E. coli* which may be transferred from contaminated hands to food and subsequently to other surfaces including food contact surfaces (Hatha et al., 2003). Our previous study already reported the biofilm-forming pathogenic *E. coli* strains from seafood pre-processing surfaces (Francis and Abdulla, 2021). The present study revealed a greater incidence of EPEC in the biofilm samples from the pre-processing environment. This is in agreement with a previous study where pathogenic serotypes of *E. coli* were reported in seafood from the retail markets of Kerala (Murugadas et al., 2016).

There are reports on the occurrence of EHEC O157 strains from shellfish and sediment samples from Cochin estuary (Antony et al., 2021) and the Indian coastal belts (Sehgal et al., 2008). Our results, however, reveal a relatively low prevalence of *E. coli* O157 serotype in the biofilm samples from the seafood contact surfaces. The presence of EHEC O157 necessitates urgent attention since it is one of the most potential pathogens responsible for severe diseases that results in hospitalizations and fatalities (Antony et al., 2021). Approximately 6.67% of the *E. coli* strains in the current study were not typeable, which indicates the presence of either novel or unrecognized O-antigen which do not react with the currently available 'O' antisera. Iguchi et al. (2017) reported that possessing a conventional 'O' serotype is not an obligate requirement to exhibit pathogenicity, as several untypeable ETEC causing diarrhea in children have been reported previously. Since *E. coli* cannot survive in the marine environment for a long time, it reinforces the idea that the detection of this organism might represent the post-harvest cross-contamination. The probability of post-harvest contamination is very high in the pre-processing centers.

Among various phylogroups, B1 and B2 were found to be the predominant phylogroups in the biofilm samples from all the food contact surfaces. This is in agreement with the findings by Dib et al. (2018) who reported phylogroup B1 is more frequently associated with fish and seafood. It has been reported that the environmental *E. coli* strains generally belong to the B1 phylogenetic group (Julian et al., 2015). Although the B1 phylogroup belongs to commensal *E. coli*, various environmental factors and horizontal gene transfer mechanisms may influence the emergence of antibiotic-resistant and virulent strains and such strains will constitute a reservoir of virulent as well as antibiotic resistant genes (Koga et al., 2014). Phylogroup B2 strains encountered in the current study are an extensive reservoir of virulent genes, which is in agreement with the findings by Koga et al. (2014), who reported that the extra intestinal pathogenic strains belong to phylogenetic group B2 that cause urinary tract infection, neonatal septicemia, or meningitis mainly. Thus, the high prevalence of B2 phylotypes of *E. coli* on seafood contact surfaces poses a serious threat to the consumer.

Kumar et al. (2001) described seafood as a potential source of STEC. STEC are also known as verocytotoxin-producing *E. coli* (VTEC) or EHEC. This pathotype is frequently implicated in food-borne outbreaks. In agreement with the findings, our results revealed the presence of toxigenic genes such as *stx1* and *stx2* in most of the *E. coli* isolates. In the present study, the most frequently encountered virulent gene combination was *stx1/stx2/eae/hly* (30%). Oh et al. (2017) reported that there was a high probability of occurrence of a combination of *eaeA*, *hlyA*, *stx1*, and *stx2*, which represented their high pathogenicity. Thus, the occurrence of these genes in *E. coli* from the food contact surfaces is a matter of concern. The high genetic diversity observed in the present study can be justified by the diverse sources of contamination of seafood through which *E. coli* enters the pre-processing environment.

## Conclusion

The results of the present study revealed that there is a considerable prevalence of virulent and MDR *E. coli* in the biofilms developed on pre-processing tables and utensils. Risk assessment of these strains revealed that they pose a significant threat to value added products that are produced from shrimps pre-processed in such facilities. Since several of the seafood pre-processing plants are located in far flung locations from the seafood



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processing plants that produce value added shrimps, time-temperature abuse of the shrimp in transit is a real possibility that will further aggravate the contaminant microbial load. In order to continue and further enhance the high value seafood export to developed markets it is essential that appropriate quality management systems such as Hazard Analysis Critical Control Point (HACCP), Total Quality Management (TQM), or Food Safety Management System (FSMS) may be adopted and executed flawlessly, in various steps involved in the processing of seafood from farm to fork.

## Author contributions

B.F. performed the experiments and prepared the manuscript; A.A.M.H supervised the work and edited the manuscript; D.P.S. conducted the statistical analysis; A.C.A. reviewed and edited the manuscript. All authors read and approved the final manuscript.

## Conflicts of interest

The authors declare that there is no conflict of interest.

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