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ISOLATION, CHARACTERISATION AND BIOFILM PRODUCTION OF *E.COLI* FROM MACKEREL *(RASTRELLIGER KANAGURTA)*

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ABSTRACT

Fish and fishery products are of great importance for human nutrition worldwide and provide clear health benefits. Fish can act as a source of food borne pathogens and may be a potential source of infection. A study was undertaken to find out the incidence and characterization of *E.coli* in fishes, determine biofilm production and antibiotic resistance of isolates. A total of 100.0 raw mackerel fish (*Rastrelliger kanagurta*) was collected from different markets in and around Puducherry. Fifty two (52.0%) isolates of *E.coli* were recovered from 100.0 samples. Among the 52.0 isolates 38.0 (73.07%) were found to produce biofilm on modified congo red agar. All the 52.0 isolates were subjected for antibiotic resistant profile test. Of the 52.0 *E. coli* isolates characterized, all (100.0%) of the isolates displayed sensitivity towards norfloxacin (10 µg), sulphametoxazole/trimethroprim (25 µg) and chloramphenicol (30 µg). On the other hand, resistance was observed against ampicilin (34.3%), tetracycline (22.5%) and Amphotericin B (10.8%). The result indicates that there is an increased prevalence of *E.coli* mainly due to contamination of the fishes during processing and also from an environment.

INTRODUCTION

The bacterial flora of marine fish, sediments and sea water has been studied all over the world. Work on fish pathogens has been carried out in marine fishes (Ward *et al*., 2009). The bacterial diseases are caused mainly due to contaminated water and sea foods (Najiah Musa *et al*., 2008). The majority of reported sea foods - associated disease outbreaks are caused by toxins; biotoxins and histamine (Chen *et al*., 2010) and viruses (Duane *et al*., 2009). India ranks second in world fish production, contributing about 5.4 percent of global fish production. Andra Pradesh, Guajarat, Karnataka, Kerala, Maharastra, Orissa, West Bengal, Tamil Nadu and Puducherry are key states that have huge potential to enhance India's seafood export. The total fish production during 2013-14 is at 9.58 million metric tonnes with a contribution of 6.14 million metric tonnes from inland sector and 3.44 million metric tonnes from marine sector respectively (Handbook on Fisheries Statistics, 2014). Over the last 25 years, the global incidence of food borne infections has markedly increased, with nearly a quarter of the population at a high risk of food borne diseases (Oliver *et al*., 2005). The World Health Organization (WHO, 2010) estimated that food borne and waterborne diarrheal diseases together kill around 2.2 million people annually. Food borne diseases always follow the consumption of contaminated food-stuffs especially from fish and animal products such as meat or carcasses contaminated with most common pathogenic bacteria like *Salmonella* spp., *Staphylococcus aureus*, *Listeria monocytogenes*, *Campylobacter* spp., *Escherichia coli* O157:H7 and *Shigella* spp (Nouichi and Hamdi, 2009). So, the routine assessment of food hygiene status is very important in food industry (Kabir, 2010). In general, Enterobacteriaceae family is a group of bacteria that is used to assess the hygienic status of food and food products among this *E. coli, Salmonella* and *Shigella* plays an important role (HPA, 2004).

Escherichia coli is a major component of the intestinal microbes in both human and animals. Colonies develop a few hours after the animal's birth, but remain inoffensive as long as they are confined to the intestinal lumen. However, in a weak or immunosuppressed people or in the case of injury to gastrointestinal barriers, even non-pathogenic strains may cause infection (Netaro and Kaper, 1996). *E. coli* strains can cause a variety of diseases, including diarrhea, dysentery,

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hemolytic uremic syndrome, and bladder & kidney infections. Different strains are usually associated with different diseases; this versatility of *E. coli* strains is due to the fact that different strains have acquired different sets of virulence genes. Human is a reservoir for *E. coli*, which can be transmitted to other human beings through the consumption of water and food contaminated by faeces (Black, 1986). The food industry has a strong focus on hygiene in order to produce safe food with high quality. The bacteria surviving after cleaning and disinfection is likely to have improved abilities to survive and also to form persistent bacterial populations (biofilm) in food production environments. (Carpentier and Cerf, 2011). The formation of microbial biofilm on the contact surfaces in the food industry is considered as an evident of health hazard. The direct contact with raw materials or foodstuffs can cause contamination and due to which the product will become unsafe (Vlkova *et al.,* 2008). In recent years, the health concern all over the world is about the emergence of numerous antibiotic resistant strains of pathogenic bacteria in foods (Cetinkaya *et al*., 2008). Among the different types of resistant mechanism, Beta-lactamases mediated drug resistance by Gram-negative bacteria is very commonly reported (Altun *et al.,* 2013). In this study we evaluated the presence of *E.coli* in fish samples, profiling of antibiotic resistance with clinical antibiotics and assessment of biofilm production.

MATERIALS AND METHODS

Collection of samples

This study period was started in March 2016 and finished at August 2016. A total of 100 raw fish samples were collected from in and around Puducherry. The sample of 50 grams of muscle part was taken for this study. All the samples were collected in sterile polyethylene bags and it was kept in the ice box. The samples were immediately transported to laboratory for further analysis.

Laboratory Analysis and Techniques

All materials needed for the study were thoroughly sterilized using a combination of the most appropriate methods of sterilization such as flaming, chemical sterilization, Ultra Violet light, hot air ovens and autoclaving to ensure that experimental materials brought in from the two major markets were not contaminated from the laboratory.

Sample preparation and isolation

The sample preparation and isolation was followed as per Gupta *et al*., 2013 with slight modification. Fifty grams of muscle part aseptically was transferred to conical flask containing 250 ml of peptone water for pre enrichment overnight at 37^0C. One ml from pre enrichment was transferred to test tubes containing 9ml Mac Conkey's broth for selective enrichment overnight at 37^0C.

Selective plating

A loopful of selective enrichment was streaked onto Mac Conkey's agar for lactose fermenting colonies. The lactose fermenting colonies were streaked onto Eosin and methylene blue agar for differentiation of metallic sheen producing colonies as per standard microbiological procedure.

Biochemical identification

The presumptive colonies were Gram's stained and subjected for primary identification tests like oxidase, catalase, indole, methyl red, voges proskauer, citrate utilisation and triple sugar iron test. Colonies also were subjected for secondary sugar fermentation tests as per standard procedures. (Veterinary Microbiology and Microbial disease, 2007)

Assessment of biofilm production of the isolates

Biofilm/Slime production assay was performed as per Dhanalakshmi *et al.* (2015). Briefly, Brain heart infusion agar supplemented with 5% sucrose and Congo red (0.08 g/l) was prepared and autoclaved. The isolates were inoculated and incubated aerobically for 24 to 48 hours. The ability of the isolates to produce bio-films was indicated by black colonies with a dry crystalline consistency. In case of negative results red colour colonies were noticed.

Antimicrobial resistance profiling of the isolates

The drug susceptibility of *E.coli* isolates was performed on Mueller Hinton agar plates by disc diffusion method in accordance with the recommendations of the Clinical and Laboratory Standards Institute (CLSI, 2012). The bacterial isolates was inoculated into nutrient broth and incubated for 18 hours at 37°C. About 0.1 ml of each bacterial isolate was seeded onto a separate Petri dish containing Mueller-Hinton agar and allowed to stand for about 5 min. The details of the antibiotics given in Table no: The interpretation of the measurement as sensitive and resistant was made according to the manufacturer standard zone size interpretative table. The percentage resistance was calculated using the formula PR=a/b×100, where 'PR' is percentage resistance, 'a' is the number of resistant isolates and 'b' is the number of isolates tested with the drug. Generally, colonies with clear zone of diameter between 0-13mm will be classified as resistant (CLSI, 2012; Akinjogunla and Enabulele, 2010).

RESULTS AND DISCUSSIONS

Overall incidence of 52.0 (52.0%) *E.coli* was recovered out of 100 samples (Table no. 1, 3 and 4). This result is higher than reported by Gupta *et al*., 2013, Punjab and kumar *et al*., 2005 Kolkata, India. Both of them reported 47.0% in a total of 96 raw fish samples. Author also cited as potential cause for *E. coli* contamination are: The quality of the ice used for conservation and also the food processing plants. According to Thampuran *et al.,* 2005, *E.coli* is commonly associated with seafood contamination in the tropics, where it is encountered in high numbers. The authors isolated *E. coli* in finfish samples acquired at the retail market in Cochin (India) and, although typical *E. coli* O157 or labile toxin-producing *E. coli* were not detected, the isolation of strains with the ability to produce hemolysis in human blood was a fact worth mentioning. Evidence from this study and according to published microbiological guidelines (Gilbert *et al*., 1996), suggests that the microbiological quality of fishes examined are unacceptable and pose a potential risk to public health. In our study, fishes harboured human disease causing bacterial organisms responsible for food poisoning especially Traveler's diarrhea. Food industry has strong focus on biofilm. The biofilm formation ability of the microbes allows withstanding extreme environmental conditions in the food industry,

especially resistant against cleaning agents. The biofilms which is present on the surfaces or equipment may cross-contaminate the food during processing (Holah *et al.,* 2004). In this study 38.0 (73.07%) out 52.0 positive isolates of *E.coli* were found to produce biofilm on modified congo red agar (Table no. 2).

sensitivity towards norfloxacin (10 µg), sulphametoxazole/ trimethroprim (25 µg) and chloramphenicol (30 µg) (Table no. 6). This result agreed by Samuel *et al.,* 2011. Who was worked in catfish and reported the same sensitivity in Malaysia. On the other hand, resistance was observed against ampicilin (34.3%), tetracycline (22.5%) and Amphotericin B (10.8%).

Table 1. Shows the growth characteristics of *E.coli* on different medium

Sl.no	Selective agar	Colony characters
1	Mac Conkey's agar (MCA)	Lactose fermenting pink colonies
2	Eosin and Methylene blue (EMB)	Metallic sheen colonies

Table 2. Shows the results biofilm on modified congo red agar

Sl. no	Selective agar	Biofilm	
		Positve (73.07%)	Negative (26.92%)
1	Modified congo red agar (MCRA)	Black colored colonies with dry crystallization	Red colored colonies were noticed

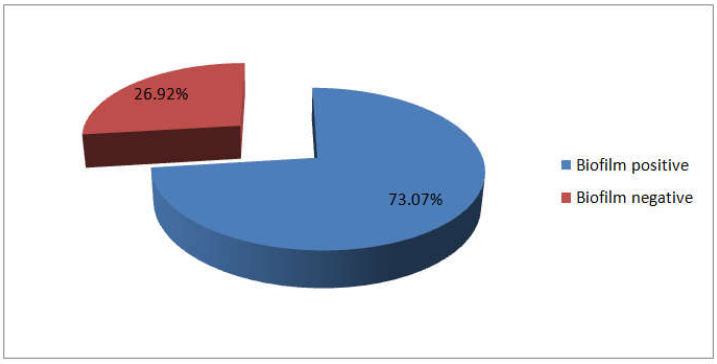


Diagram 1. Pie diagram represents the results of biofilm (n=52)

These isolates were given strong black color formation on congo red agar. This result is lower than Dhanalakshmi *et al*., 2015 report. Who reported 100% biofilm production of *E.coli* isolates on modified congo red agar in Namakaakl, Tamil nadu. Out of 14.0 *E.coli* isolates tested by Dadawala *et al*., 2013 in Gujarat 12.0 were found to produce black colonies within 24-48 hr. The remaining 2 isolates failed to produce black colonies even after 72hrs of incubation. The observation of slime production in *E.coli* seems to be novel and this can be taken up as a qualitative assessment of biofilm, Such slime production has been reported and documented by Aricola *et al*., 2001; Vasudevan *et al*., 2003 on *Staphylococcus aureus.* Slime productions reflect the capacity of bacteria to.adhere specific host tissues and thereby to produce invasive microcolonies (Baselga *et al*., 1994).

Table 3. Shows the results of primary identification tests

Test	Result
Gram's staining	Negative
Shape	Medium sized bacilli
Oxidase	Negative
Catalase	Positive
Triple sugar iron	Acid butt, acid slant and gas production
Indole	Positive
Methyl red	Positive
Voges proskauer	Negative
Citrate utilization	Negative
Urease	Negative

The biofilm forming isolates are resistant to antimicrobial agents due to the lack of penetration of antimicrobial agents (Dhanalakshmi *et al.,* 2015). In this study, 52.0 isolates of *E. coli* were isolated from fish samples. Of the 52.0 *E. coli* isolates characterized, all (100.0%) of the isolates displayed

Table. 4 Shows results of secondary identification tests

Tests	Results
Arabinose	Positive
Lactose	Positive
Maltose	Positive
Rhamnose	Positive
Trehalose	Positive
Xylose	Positive
Adonitol	Negative
Sucrose	Negative
Cellobiose	Negative
Inositol	Negative

Table 5. List of antibiotics used in this study

S.no	Antibiotics	Content / disk
1	Ampicillin	25µg
2	Amphotericin B	10 units/disk
3	Cephalothin	30µg
4	Cefoxitin	30µg
5	Cefriaxone	30µg
6	Ciprofloxacin	10µg
7	Chloramphenicol	30µg
8	Gentamicin	120µg
9	Imipenem	30µg
10	Norfloxacin	10µg
11	Nalidixic acid	30µg
12	Sulfamethoxazole- trimethoprim	25µg
13	Tetracycline	30µg

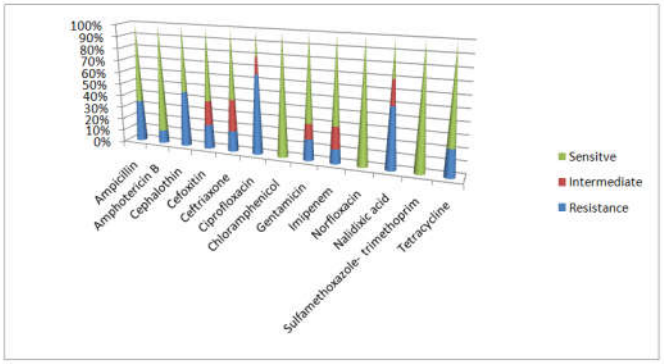
The assertive that commercial fish and seafood may constitute repositories of multi resistant bacteria to antibiotics was reported by Ryu *et al*., 2012. The authors also found high index of resistance to tetracycline (30.7%) in South Korea. Many studies have reported almost same results concerning

Tetracycline and Ampicillin resistant (Soltan *et al.,* 2007; Bywater and Deluyker, 2004). Similarly, Tansuphasiri *et al*. (2006) evaluated 239 isolates of enterococci (113 from frozen foods and 126 from environmental water) for their resistance to 8 antibiotics by agar disk diffusion method.

Table 6. Results of antibiotic resistance profile of the isolates

S.no	Antibiotics	Results (%)		
		R	I	S
1	Ampicillin	34.3	0	65.7
2	Amphotericin B	10.8	0	89.2
3	Cephalothin	45.7	0	54.3
4	Cefoxitin	20.0	20.0	60.0
5	Cefriaxone	17.0	26.0	57.0
6	Ciprofloxacin	66.0	14	20.0
7	Chloramphenicol	0	0	100.0
8	Gentamicin	17.5	12.5	70.0
9	Imipenem	12.0	18.0	70.0
10	Norfloxacin	0	0	100.0
11	Nalidixic acid	50.0	20.0	30.0
12	Sulfamethoxazole-trimethoprim	0	0	100.0
13	Tetracycline	22.5	0	77.5

R – resistance, I- intermediate, S- sensitive



Graph. 1 Represents the results of antibiogram

Most isolates from both sources were resistant to tetracycline (64.1% food strains; 46.8% water strains) and ciprofloxacin (53.4% food strains; 48.4% water strains). A relatively high prevalence of chloramphenicol and trimethoprim-sulfamethoxazole resistance was present, ranging from 9.7 to 27.2% for food strains and 10.3 to 15.9% for water strains. Evolution of bacteria towards resistance has been considerably accelerated by the selective pressure exerted by over prescription of drugs in clinical settings and their heavy use as growth promoters for farm animals such as fish. When antimicrobial drugs are administered to food animals, they can thus promote the emergence of resistance in bacteria that may not be pathogenic to the animals, but are pathogenic to humans (Bates, 1994; Piddock, 1996).

Conclusion

Detection of *E.coli* is an indication of contamination during handling and processing of raw fishes. Survival of these organisms in fresh fish is likely to cause menace to consumer health and also chances for quick spoilage. Proper cooking may reduce the risk of contacting the infections. This study clearly indicating the biofilm producing ability of the *E.coli* isolates. Biofilm isolates will contaminate the handling equipment also resistance occurs against the disinfectant agents. Biofilm also increases the antibiotic resistance. Results of the study give an insight to the necessity to implementing principles of Hazard analysis critical control point (HACCP) during the production of food in order to safeguard consumer health.

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