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MOLECULAR IDENTIFICATION OF FOOD-BORNE PATHOGENIC *ESCHERICHIA COLI* FROM MARINE FISHES

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ABSTRACT

The collection and identification of bacterial isolates are paramount for the correct diagnosis and from different food-borne diseases. The methods of bacterial identification relied on phenotypic tests, which are often time-consuming and unreliable when identifying atypical strains. Traditional methods could be done and followed by a molecular approach which is fast and reliable for the identification of isolates, this could be achieved by comparing a 16S ribosomal RNA gene sequence with publicly available sequences. In the present study, isolated bacteria were characterized and its DNA was extracted and amplified by PCR using universal primers, and then a partial 16S rRNA gene sequence was obtained and compared with sequences deposited in public DNA sequence databases; EMBL/GeneBank and DDBJ. Bacterial isolates belonging to *Escherichia coli* species as identified by culturing methods, biochemical tests as well partial 16S rRNA gene sequence to the species level. We conclude that the identification of bacteria with partial 16S rRNA gene sequence is an efficient and specific tool complement to phenotypic identification.

KEYWORDS: Bacteria*, Escherichia coli,* Molecular identification; universal primer; 16S rRNA gene

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INTRODUCTION

Investigation of the incidence and virulence proprieties of *Escherichia coli* in marine fishes is an important research due to the increasing consummation of seafood worldwide to access the cause, source of contamination and quality of seafood. Due to their immunity and healthy, among children in developing countries, *E. coli* is the main etiologic cause of diarrhea and represents a major public health problem in these areas. [1,2] One group of *E. coli* organisms which have an important role as a cause of the enteric and diarrheal disease is diarrheagenic *E. coli* (DEC).[2] On the basis of distinct epidemiological and clinical features, specific virulence determinants, and association with certain serotypes[3,4],as well as their prevalence in several different geographical areas.[3,5]*E. coli* in fish is considered as an indicator of potential sewage pollution. Levels of it are used to determine whether local beaches should be posted with "no water contact" advisories. There are a variety of types of *E. coli*. [6] *E. coli* is a bacterium that commonly lives in the intestine of people, animal, and fish. There are many strains (types) of *E. coli*. Most of them are normal inhabitants in the small intestine and colon and are non-pathogenic, meaning they do not cause disease in the intestine. Nevertheless, these non-pathogenic *E. coli* can cause disease if they spread outside the intestine. The pathogenic strains of E. coli may cause diarrhea by producing and releasing toxins (called enterotoxigenic *E. coli* or ETEC) and cause of food in fish.[7]The intestinal pathogens are also known as diarrheagenic *E. coli* (DEC) of which six categories have been characterized: enteropathogenic *E. coli* (EPEC), Shiga-toxin-producing *E. coli* (STEC) or verocytotoxin producing *E. coli* (VTEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), and diffusely adhering *E. coli* (DAEC).[8,10] The extraintestinal pathogens (EXPEC) are more prevalent strains include those associated with urinary tract infections (UPEC), neonatal meningitis (MAEC), and bacteremia. Lipopolysaccharide (LPS) is a key component of the outer membrane of Gram-negative bacteria. It commonly comprises three distinct regions: the proximal hydrophobic lipid A region, the distal hydrophilic O antigen, and the interconnecting core oligosaccharide. The O-antigen, which consists of many repeats of an oligosaccharide (O unit), is one of the most variable cell constituents due to the variations in the types of sugars present, the arrangement of the sugars within O unit, and the linkages between O units.[11,12]The majority of reported sea foods - associated disease outbreaks are caused by toxins; biotoxins and histamine and viruses.[13,14] Bacterial flora of marine fish, sediments, and seawater have been studied the world over with a view to explain the spoilage of fish.[15] Immediately after the procurement, there is a significant amount of data on the microbiology of seafood produced or imported in different countries.[16] Environmental degradation of the coastal area of Chennai (Ennore creek) affecting fish and fisherfolk has already been reported.[17] The aims of this study were to determine to isolate, to characterize via culturing method, biochemical tests, PCR methods *E. coli* associated with marine fishes.

MATERIAL AND METHODS

Isolation of E. coli from marine fishes

Marine fish samples were collected from the local fish market in Thanjavur,Tamil Nadu. Four different species marine fishes were collected including *Sardinella longiceps, Rastrelliger kanagurta, Epinephelus tauvina* and *Scomberomorus guttatus*. To examine the bacterial organisms in the fishes taken for study, the methods of culture and plating as described by Venkataraman and Sreenivasan (1952) were followed. The tissues of *S. longiceps, R. kanagurta, E. tauvina* and *S. guttatus* were dissected and processed. The 25g portions were cut, homogenized with 225ml peptone saline water and after serial dilution, they were subjected to the separate culture medium.

Media

Culture technique procedures recommended by Bergey (1948) were followed. The media employed for the isolation of *E. coli* were nutrient agar, nutrient broth, Mac Conkey agar and hicrome UTI agar and these were time tested ones in bacterial cultures.

Biochemical Characterization

To identify and characterize the isolated pathogens, biochemical parameters such as test for oxidase, catalase, indole, methyl red, Vp citrate, TSI, urease and nitrate reduction were carried out following the standard process in the biochemical analysis.

DNA Isolation

DNA from the bacterial genome was extracted as per standard Proteinase-K digestion method.[18] Bacterial cultures were prepared and suspended in Luria-Bertani broth (Hi-Media, India) and incubated at 37 °C, 110 rpm for 12 hours. The 12-hour old bacterial cells were pelleted at 15000 × g for 10 minutes and then suspended in TEN (Tris-HCl (pH 7.2), 10 mM EDTA, 250 mM NaCl) buffer having 1% sodium dodecyl sulfate (Hi-Media, India). Proteinase-K(Hi-Media, India) was then added to a final concentration of 100 µg/ml and mixed gently. The suspension was incubated at 37 °C for 60 min. DNA obtained by sequential phenol-chloroform and chloroform-isoamyl alcohol extractions was precipitated by adding 2.5 volumes of absolute ethanol, and DNA was suspended in 100 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA-pH 7.5). DNA was checked for purity by agarose gel electrophoresis.

PCR amplification of 16S rRNA gene

PCR reaction was performed in a gradient thermal cycler (Eppendorf, Germany). The universal primers (Forward primer 5'-AGAGTTTGATCMTGGCTCAG-3' and reverse primer 5'-GGTTACCTTGTTACGACTT-3') were used for the amplification of the 16S rRNA gene fragment. The reaction mixture of 50 µl consisted of 10 ng of genomic DNA, 2.5 U of Taq DNA polymerase, 5 µl of 10X PCR amplification buffer (100 mM Tris-HCl, 500 mMKCl pH-8.3), 200µM dNTP, 10 p moles each of the two universal primers and 1.5mM $MgCl_2$. Amplification was done by initial denaturation at 94ºC for 3 minutes, followed by 30 cycles of denaturation at 94ºC for 30 seconds, the annealing temperature of primers was

55ºC for 30 second and extension at 72ºC for 1 minute. The final extension was conducted at 72ºC for 10 minutes. Agarose gel electrophoresis of PCR product was carried out as follow 10 µl of the reaction mixture was then analyzed by submarine gel electrophoresis using 1.0 % agarose with ethidium bromide at 8V/cm and the reaction product was visualized under gel documentation system.The PCR products are subjected to Sanger sequencing, where Sequence analysis has been performed using Bioedit software. BioEdit is a biological sequence analysis / alignment bioinformatics tool developed for windows. An intuitive multiple document interfaces with suitable features create alignment and manipulation of sequences relatively simple on our desktop computer.

RESULTS

Table 1
Marine fishes used in this study

S.No.	Common Name	Local Name	Scientific Name
1	Indian oil sardine	Mathi Meen	*Sardinella longiceps*
2	Indian mackerel	Aiyla Meen	*Rastrelliger kanagurta*
3	Greasy grouper	Punni-calawah	*Epinephelus tauvina*
4	Indo-Pacific king mackerel	Vanjaram meen	*Scomberomorus guttatus*

Table 2
Prevalence of E. coli in marine fishes obtained from Thanjavur fish market

FISH	*E. coli*
Sardinella longiceps	+
Rastrelliger kanagurta	+
Epinephelus tauvina	+
Scomberomorus guttatus	+

+: positive

Table 3
Biochemical study on bacteria isolates

Biochemical Tests	*E. coli*
Gram staining	-
Catalase	+
Oxidase	+
MR	-
VP	+
Indole	-
Citrate utilization	+
TCI	+
Lactose fermentation	+
Glucose fermentation	+/-
Sucrose fermentation	+
Mannitol fermentation	+/-

+: positive test -: negative test

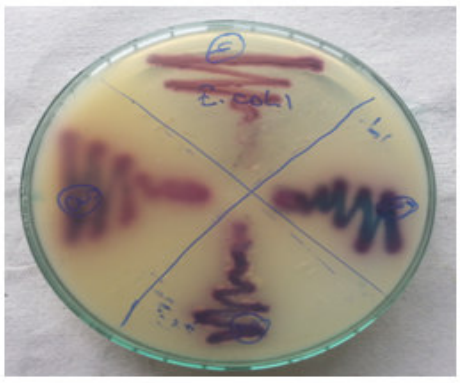
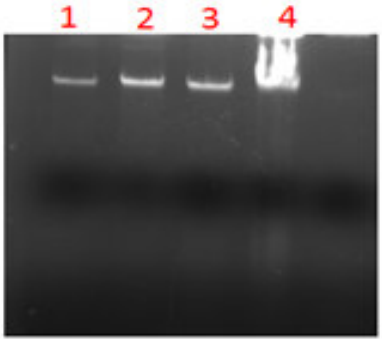
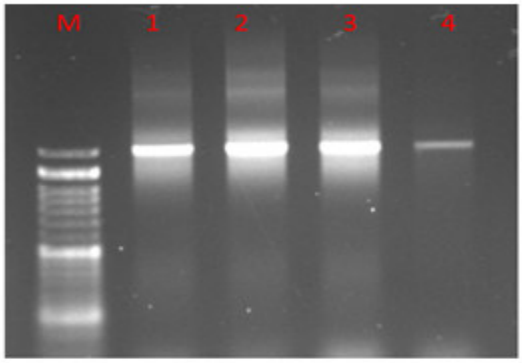


Figure 1
Escherichia coli on hicrome uti agar plate.



Lane 1: Genomic DNA from isolate 1
Lane 2: Genomic DNA from isolate 2
Lane 3: Genomic DNA from isolate 3
Lane 4: Genomic DNA from isolate 4

Figure 2
Genomic DNA quality from agarose gel electrophoresis



Lane M: marker
Lane 1: isolate one
Lane 2: isolate two
Lane 3: isolate three
Lane 4: isolate four

Figure 3
Agarose gel electrophoresis showing PCR amplification of 16S rRNA gene from four isolates using universal primers

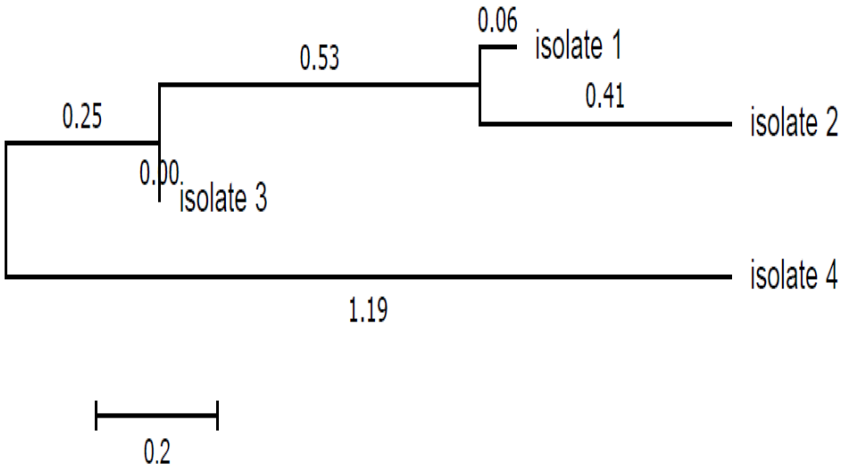


Figure 4
Evolutionary relationships of different samples using Mega 6 version phylogenetic analysis tool.

In the present study, four different fish species were used for the isolation of *E. coli* bacteria, where culturing as well biochemical tests were performed for the identification and characterization of isolates, Partial 16S ribosomal RNA gene sequences were obtained and checked, in order to ascertain whether identification of bacterial species would be possible through comparison with publicly available sequences with the BLAST algorithm in GeneBank/EMBL/DDBJ DNA databases, and with the Sequence match algorithm in the RDP-II database. All selected marine fishes showed the presence of *E. coli*. The incidence of *E. coli* bacteria isolated in different marine fishes is observed in our study (Table 2). The selective media used Hicrome UTI agar showed the presence of the *E. coli* bacteria pink-purple colonies are observed on hicrome UTI agar plates (Fig. 1). The isolates were presumptively identified by biochemical profiling the different reactions and different changes in the media due to the behavior of isolated bacteria to the subjected media and chemicals present in media different changes were observed and compared to the standards records there is confirmation of *E. coli* (Table 3). Genomic DNA was isolated and characterized by standard Proteinase-K digestion method, and its quality was checked using agarose gel methods (Fig.2), PCR was performed using universal primers 27F and 1492R the products were observed as 1500bp (Fig.3). PCR products were subjected to the Sanger sequencing were the results were analyzed using BIOEDIT as well MEGA6 software Phylogenetic tree was constructed using neighbor-joining algorithm of nucleotides sequence of 16S rRNA gene. The number in the branch of phylogram indicates bootstrap value (%) by 2000-replication multiple and scale indicates one per 2000 substitutions of nucleotides sequence of 16S rRNA gene (Fig.4). All isolates have the same root isolate one and two are closely related and isolate 3 is closely related to isolate1 and 2 at the last the isolate 4 is fall from isolate 1, 2 and 3.

DISCUSSION

A food-borne disease is a worldwide problem. Recent development in food production and processing techniques and the subsequent changing trends in food consumption have resulted in the emergence of new hazards. The increased contact among different nations has stimulated rapid global distribution of foods as well as food-borne pathogens. *E. coli* is common bacteria found in food, especially seafood in an environment in different conditions[18], and plays a big role in economic losses food industry. Therefore, the present study was carried out to isolate *E. coli* in different fish species of and identifying it by different methods. From the selected fish samples in local market, all showed the presence of the targeted bacteria. In different studies the incidence of *E. coli* samples collection was done from different localities to give a good chance for isolation of different *E. coli* bacteria and for determination greater incidence in different food materials, in our study the sample collection was done in local market targeting different marine fish species available. The isolation of different pathogens in high numbers indicates there wide prevalence and public health Verocytotoxigenic *E. coli* (VTEC) emerged as a serious food borne threat to public health in the latter part of the 20th century.[19] Germani noted that *E. coli* incidence differ from area to another area according to the demographic and socioeconomic characteristics, environmental factors, and type of food introduced to human, animals and fish and susceptibility of fish species,[20] in our study we did not focus on the source of the sample. After isolation and biochemical identification, all isolates were subjected to detailed genotypic and phenotypic characterizations. All the 4 isolates were positive test for Indole, methyl-red test, catalase and for lactose fermenting, and negative biochemical test are Voges-Proskuaer, citrate, Urease, there we confirmed that these organism are *E. coli*, the amplification of 16s rRNA gene is another factor which confirm that the isolates are bacteria.[20] Genomic DNA was extracted from the isolated bacterial. 16S rRNA gene was amplified and sequenced by using their respective primers. A total of 1500 bp of the 16S rRNA gene were sequenced and used for the identification of isolated bacterial strain.

CONCLUSION

This study revealed an overall incidence of *E. coli* in marine fishes which clearly indicate that in the study area fishes do harbor a number of food-borne pathogens. Isolates of pathogens expressed different traits indicating that prevalent pathogens were capable of inducing disease in consumers. Finally, the results of the present study indicated possible risks to consumers of marine fishes in the region that demand action to address this public health concern.

CONFLICT OF INTEREST

Conflict of interest declared none.

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