Indian Journal of Geo Marine Sciences
Vol. 52 (08), August 2023, pp. 363-370
DOI: 10.56042/ijms.v52i08.11019

Detection of *Escherichia fergusonii* - an emerging pathogen harbouring drug resistant genes from seafood samples of Tamil Nadu, India

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Received 18 January 2022; revised 29 July 2023

Escherichia fergusonii harbouring drug resistant genes was isolated from seafood samples of Tamil Nadu, India while attempting to isolate *Escherichia coli*. Isolation of STEC was done by enrichment and plating on MacConkey agar MUG and sorbitol negative colonies were confirmed by IMViC test and by an automated system (Vitek-2 compact, BioMerieux, France). Antibiotic sensitivity test was performed by disc diffusion method and by PCR for antibiotic resistance genes. A total of 302 presumptive *E. coli* was isolated, of which 126 isolates were confirmed as *E. coli*. Few isolates could not ferment sorbitol and produce β-glucuronidase, and these were confirmed as presumptive *E. coli* O157:H7. However, two isolates were positive for adonitol and therefore confirmed as *E. fergusonii* (E011 and E060). The two isolates were sorbitol and lactose negative and adonitol, amygdalin and cellobiose positive. The isolates could not produce the enzyme β-glucuronidase. The isolates were then confirmed as *E. fergusonii* by gene specific PCR assay and 16S rDNA sequencing. ESBL studies showed that both the isolates harbour bla_{TEM}. Presence of drug resistance and ESBL in *E. fergusonii* isolated from seafood samples is a matter of public health concern, as the resistance could enter the food chain and hinder the effectiveness of drugs used for treatment. To the best of our knowledge, this is the first report on the occurrence of *E. fergusonii* harbouring drug resistance genes from seafood samples of Tamil Nadu, India.

[**Keywords**: Antibiotic resistance, Drug resistance, ESBL, *Escherichia fergusonii*, Public health, Seafood]

Introduction

Escherichia fergusonii is an emerging pathogen, first isolated in 1985 from human blood[1]. This was initially known as Enteric Group 10 (EG 10). It exhibits characters biochemically distinct from members of the family *Enterobacteriaceae* and shows 64 % similarity to *Escherichia coli* by DNA hybridization[2]. This bacterium has been isolated from blood, urine, abdominal wounds, gall bladder fluid, faecal material of human, goat, sheep, turkey, chicken, pigs and cattle[1,3-7]. There are also reports on the occurrence of *E. fergusonii* in humans[4,8,9] causing bacteraemia, urinary tract infection and diarrhoea. Biochemically, *E. fergusonii* could be differentiated from *E. coli* by its inability to ferment sorbitol and lactose. The inability of *E. fergusonii* to ferment sorbitol and produce β-glucuronidase is the two biochemical characteristics shared with *E. coli* of the serotype O157:H7(ref. 1). However, *E. coli* O157:H7 does not use adonitol or citrate as the primary source of carbon and this specific biochemical character is used as a distinguishing character for the confirmation of *E. fergusonii*[3].

Generally, antibiotics are used to curb infections caused by bacteria. Prolonged use of antibiotics has led to the emergence of resistant strains of bacteria. The rise in multidrug resistance in Gram-negative bacteria poses a major challenge in developing countries. The antibiotics used for the treatment of *E. coli* are prescribed for *E. fergusonii* as well[10,11]. However, of late antibiotic treatment has been hampered due to the antibiotic resistance genes possessed by this bacterium[12-14]. Resistance in Gram-negative bacteria against β-lactam antibiotics is mainly mediated via the production of Extended Spectrum β-Lactamases (ESBL), ampC β-lactamases and carbapenemases[15]. Studies on *E. fergusonii* are gaining importance lately due to their multi-drug resistance possessing extended β-lactamase gene[12,16-18]. This is the first report on the detection of *Escherichia fergusonii* harbouring drug resistance genes from seafood samples of Chennai. This attempt of isolation of *E. coli* O157:H7 from market samples of Old Mahabalipuram Road (OMR), Chennai resulted in the isolation of *E. fergusonii*, as this bacterium also does not utilize sorbitol and produce β-glucuronidase, a

key character of *E. coli* O157:H7. The isolates were confirmed by gene specific primer and 16S rDNA sequencing and its characteristics of drug resistance has also been studied.

Materials and Methods

Isolation and biochemical confirmation of *E. fergusonii*

Fish (*Nemipterus japonicus* (Bloch, 1791)) and shellfish (*Penaeus indicus* Milne-Edwards, 1837) were collected from three fish markets of Old Mahaballipuram Road (OMR), Chennai namely Kovalam, Kelambakam and Navallur fish markets for one year *i.e.* from July 2018 to June 2019. Three tube MPN method was used for the quantification of total and faecal coliforms[19]. Samples were collected in sterile containers and transported immediately to the laboratory for analysis. Ten gram of the sample was homogenized with 90 ml of saline (0.85 % NaCl) in a sterile blender and inoculated into Lauryl Sulphate Tryptose Broth (LSTB) and later incubated at 37 °C for 24 h. The LSTB tubes indicating turbidity and gas production in Durham tubes were considered positive for total coliform of which two loopful of broth culture from positive LSTB tubes were inoculated into corresponding labelled tubes containing 5 ml of *E. coli* (EC) broth. EC tubes which developed turbidity and gas production following 24 h incubation at 44.5 °C was confirmed as faecal coliforms. For the isolation of *E. coli*, a loop of broth from positive EC broth tubes was streaked onto Eosin Methylene Blue (EMB) agar plates and Sorbitol MacConkey Agar plates (SMA). A minimum of five typical colonies were picked from EMB and SMA and streaked on Tryptone Soya Agar (TSA) plates to obtain pure cultures and subjected to IMViC test for the confirmation of *E. coli*[19].

Isolation of Shiga toxin producing *E. coli* (STEC) was attempted with enrichment of 25 g of homogenized fish sample in 225 ml modified EC broth containing sodium novobiocin (20 mg/L) for 18 h at 37 °C. The enriched sample was streaked on MacConkey agar supplemented with cefixime (0.05 mg/l) and potassium tellurite (2.5 mg/l) and incubated at 37 °C for 24 h. Sorbitol negative colonies were checked for the absence of methyl umberlliferyl-β-glucuronide (MUG) reaction by plating on MUG agar (Himedia, Mumbai). MUG and sorbitol negative colonies were confirmed by IMViC test. The isolates were further confirmed by an automated system (Vitek-2 compact, BioMerieux, France).

Confirmation of *E. fergusonii* by molecular methods

DNA was extracted by standard procedure[20]. Single bacterial colony was suspended in 567 µl of 1 × TE buffer (10 mMTrisHCl, 1 mM EDTA, pH 8.0), 30 µl of 10 % sodium dodecyl sulphate and 3 µl of proteinase K (20 mg ml^{-1}), and incubated at 57 °C for 1 h. Then, 100 µl of 5 M NaCl and 80 µl of 10 % Cetyltrimethyl Ammonium Bromide (CTAB) in 0.7 M NaCl was added, the solution was mixed and incubated in a water bath at 65 °C for 10 min. DNA was extracted with 800 µl of phenol:chloroform:isoamyl alcohol (25:24:1). The aqueous layer was collected in a fresh micro centrifuge tube and the DNA was precipitated by adding 0.6 volume of isopropanol. DNA was pelleted by centrifugation at 10,000 × g for 10 min using a refrigerated centrifuge (Sigma, USA). The DNA pellet was washed with 70 % ethanol, air dried and suspended in 100 µl of TE buffer.

The universal primer (5'-AGAGTTTGATCCTGGCTCAG -3' and 5'-GGTTACCTTGTTACGACT-3')[21] was used for the amplification of 16S rDNA gene. A putative transcriptional activator for a multiple antibiotic resistance gene specific for *E. fergusonii* (EFER_1569) (EFER YPF: 5' GCAATATACAGGACACAGTGTCG 3', EFER YP-R: 5' CTATGAAGGGAAGGGTAGGAGC 3') (432 bp)[22] was also used for the confirmation of *E. fergusonii*. The amplified PCR products were electrophoresed on 1 % agarose gel, stained in ethidium bromide and photographed in a gel documentation system. The amplified product of Universal primer and EFER_1569 were sequenced. The sequences were edited using Bioedit software v 7.0.9. The edited sequences were compared with GenBank Nucleotide Database (http://www.ncbi.nlm.nih.gov/BLAST/) using the algorithm Blast N[(ref. 23)]. The output of BLAST searches was sorted based on maximum identity with other genus or species names in GenBank records. The gene sequences of the bacterial isolate were deposited in GenBank and accession numbers were obtained.

Antibiotic sensitivity test by disc diffusion method

Antibiotic susceptibility test was performed by the Kirby-Bauer disk diffusion method on Muller-Hinton agar[24]. Bacterial culture was grown on Trypticase soya broth (Himedia, Mumbai) to 10^8 cells ml^{-1}, and seeded on Mueller-Hinton agar as a uniform lawn.

Antibiotic discs, ampicillin (30 µg), amikacin (30 µg), cefotaxime (30 µg), colistin (10 µg), chloramphenicol (30 µg), cefoxitin (30 µg), imipenen (10 µg) and trimethoprim (5 µg) from Himedia (Mumbai, India) were used. The plates were incubated at 30±2 °C for 24 h and the diameter of the zone of inhibition was measured and compared with zone diameter interpretative chart to determine the sensitivity of the isolates to the antibiotics[25].

PCR for the detection of drug resistance genes

For the detection of antibiotic resistant genes, eight different primers were used. The details of primers and annealing temperature are given in Table 1 (Sr. Nos. 1 – 8). To understand whether *E. fergusonii* had acquired the virulence genes from *E. coli* O157:H7, shiga toxigenic virulence genes *stx1*, *stx2*, *eae* and *hlyA* were studied[26] with primers as given in Table 1 (Sr. Nos. 9 – 12).

Results and Discussion

Seventy two samples comprising 36 fish and 36 shrimp samples were collected from three fish markets of OMR, Chennai *viz*., Kovalam, Kelambakam and Navallur fish markets for one year. Total coliform bacteria were high ranging from MPN 450 to > 1400 /10 g, followed by faecal coliforms from 25 to > 1400 MPN/10 g and *Escherichia coli* from MPN 4 to 1100 /10 g of sample indicating faecal contamination as these organisms are not part of the normal microflora of fish. A total of 302 presumptive *E. coli* isolates from EMB agar (colonies showing metallic sheen) and Sorbitol MacConkey agar (both fermentative and non-fermentative colonies) were isolated, from which 126 isolates were confirmed as *E. coli* and two as *E. Fergusonii* (E011 and E060) by biochemical tests. The two isolates of *E. fergusonii* were sorbitol and lactose negative and adonitol, amygdalin and cellobiose positive[1] (Table 2). The isolates could not produce the enzyme β-glucuronidase on MUG agar (Himedia, Chennai). Based on the inability of the two isolates to ferment sorbitol and produce β-glucuronidase, the isolates were initially confirmed as *E. coli* O157:H7 as these two traits are the common biochemical characters that differentiate *E. coli* from *E. coli* O157:H7[1,27]. However, the these two isolates were later found positive for adonitol and therefore confirmed as *E. fergusonii*[1,3]. *E. coli* does not use adonitol or citrate as the primary carbon source[3]. *Escherichia fergusonii* isolates E011 and E060 were isolated from shrimp samples of Kelambakkam market in the month of July and December 2019, respectively.

Table 1 — Details of primers used in the study

S. No.	Target gene	Primer sequence (5´ - 3´)	Annealing temperature (°C)	PCR product size (bp)	Reference
1	bla_{TEM}	ATGAGTATTCAACATTTCCG CCAATGCTTAATCAGTGA GG	60	850	Maneesh *et al.*[49]
2	bla_{SHV}	TCAGCGAAAAACACCTTG TCCCGCAGATAAATCACCA	60	475	
3	$bla_{CTX\text{-}M\text{-}1}$	GACGATGTCACTGGCTGAGC AGCCGCCGACGCTAATACA´	55	499	
4	AmpC	CCCCGCTTATAGAGCAACAA TCAATGGTCGACTTCACACC	60	634	
5	*sul1*	CGGCGTGGGCTACCTGAACG GCCGATCGCGTGAAGTTCCG	60	433	Kerrn *et al*.[50]
6	*sul2*	GCGCTCAAGGCAGATGGCATT GCGTTTGATACCGGCACCCGT	60	293	
7	Integron	GCCACTGCGCCGTTACCACC GGCCGAGCAGATCCTGCACG	60	898	
8	bla_{NDM}	GGTTTGGCGATCTGGTTTTC CGGAATGGCTCATCACGATC-	52	621	Sheikh *et al*.[51]
9	*stx1*	ATAAATCGCCATTCGTTGACTAC AGAACGCCCACTGAGATCATC	53	180	
10	*stx*2	GGCACTGTCTGAAACTGCTCC TCGCCAGTTAATCTGACATTCTG	53	255	
11	*eae*	GACCCGGCACAAGCATAAGC CCACCTGCAGCAACAAGAGG	53	384	
12	*hlyA*	GCATCATCAAGCGTACGTTCC AATGAGCCAAGCTGGTTAGCT	53	534	

Table 2 — Biochemical characterization of isolates of *E. fergusonii*

S. No	Biochemical test	E011	E060	S. No	Biochemical test	E011	E060
1	Ala-Phe-Pro-Arylamidase	-	-	25	Saccharose/Sucrose	-	-
2	Adonitol	+	+	26	D-Tagatose	+	+
3	L-Pyrrolydonyl-Arylamidase	+	+	27	D-Trahalose	+	+
4	L-Arabitol	-	-	28	Citrate (Sodium)	-	-
5	D-Cellobiose	+	+	29	Malonate	-	-
6	Beta-Galactosidase	+	+	30	5-Keto D-Gluconate	-	-
7	H_2S Production	-	-	31	L-Lactate alkalinisation	+	+
8	Beta-N-Acetyl-Glucosaminidase	-	-	32	Alpha-Glucosidase	-	-
9	Glutamyl Arylamidase pNA	-	-	33	Succinate alkalinisation	+	+
10	D-Glucose	+	+	34	Beta-N-Acetyl-Galactosaminidase	-	-
11	Gamma-Glutamyl-Transferase	-	-	35	Alpha-Galalctosidase	-	-
12	Fermentation/Glucose	+	+	36	Phosphatase	(-)	(-)
13	Beta Glucosidase	-	-	37	Glycine Arylamidase	-	-
14	D-Maltose	+	+	38	Ornithine Decarboxylase	+	+
15	D-Mannitol	+	+	39	Lysine Decarboxylase	+	+
16	D-Mannose	+	+	40	L-Histidine assimilation	-	-
17	Beta-Xylosidase	-	-	41	Coumarate	+	+
18	Beta-Alanine-Arylamidase pNA	-	-	42	Beta-Glucoronidase	-	-
19	L-Proline Arylamidase	+	+	43	O/129 Resistance	+	+
20	Lipase	-	-	44	Glu-Gly-Arg-Arylamidase	-	-
21	Palatinose	-	-	45	L-Malate assimilation	-	-
22	Tyrosine-Arylamidase	+	+	46	Ellman	+	+
23	Urease	-	-	47	L-Lacatate assimilation	-	-
24	D-Sorbitol	-	-				

PCR primers specific to conserved genes in *E. fergusonii* was also performed for confirmation of *E. fergusonii*. The primers designed to generate an amplicon size of 432 bp were used in this study. Both the *E. fergusonii* isolates produced the expected amplicon size of 432 bp and confirmed as *E. fergusonii*[22] (Fig. 1).

DNA from *E. fergusonii* (E011) was amplified using universal 16S rRNA and the product was sequenced. For comparing the sequence, Basic Local Alignment Search Tool (BLAST) was used and *E. fergusonii* (E011) showed 99 % homology to *E. fergusonii*. The accession sequences of *E. fergusonii* are submitted to the National Centre for Biotechnology Information (NCBI) with accession numbers MN900568 and MN817666.

Escherichia fergusonii shares several phenotypic traits with serotype O157:H7 of *E. coli*, such as an inability to ferment sorbitol and failure to produce β-glucuronidase. *Escherichia fergusonii* isolate that can react with antisera specific for the *E. coli* O antigen 157 has been reported by Fegan *et al*.[28]. Further, both intraspecies and interspecies horizontal gene transfer of O antigen gene clusters in *E. coli* and

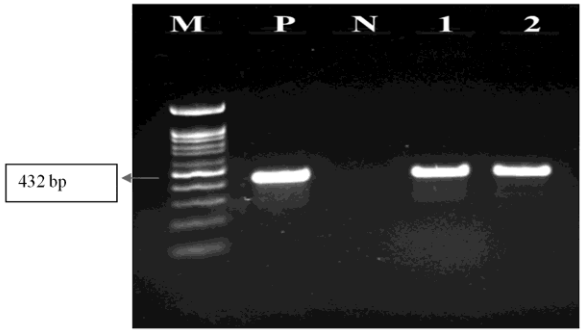

Fig. 1 — Gene specific PCR amplification of *Escherichia fergusonii* (lane 1 – 100 bp DNA ladder, lane 2 – positive control (clinical *E. fergusonii*), lane 3 – negative control, lane 4 – E011, lane 5 – E060)

S. enterica have been reported by Reeves & Wang[29]. Therefore, to understand whether *E. fergusonii* could have acquired the virulence factors of shiga toxin from *E. coli* O157:H7, shiga toxigenic virulence genes *stx*1, *stx*2, *eae* and *hly*A were studied by PCR. However, the results concluded that *E. fergusonii* did not carry the shiga toxigenic genes of *E. coli* O157:H7.

Antibiotic resistance profile of *E. fergusonii* isolates by disc diffusion method from the present study revealed resistance of these isolates to antibiotics (Table 3). The

isolate E060 showed resistance to five of the eight antibiotics studied. Isolate E060 was resistant to ampicillin, cefotaxime, chloramphenicol, cefoxitin and trimethoprim (Table 3). Antimicrobial resistance of *E. fergusonii* has been reported earlier[13,14,30] and multiple antibiotic resistance of *E. fergusonii* has also been reported in earlier studies[2,12]. Comparative antibiotic studies on *E. fergusonii* and *E. coli* from dairy cattle with diarrhoea from Turkey have concluded that *E. fergusonii* has developed resistance to penicillin G and erythromycin[30]. Studies on *E. fergusonii* from non-human primates in Africa have reported 87 % resistance to polymixin B, colistin and carbapenem group[31], however, the antibiotic resistance by disc diffusion method could not be compared with this study as the antibiotics used in current study are different from those used in earlier studies. Based on the previous studies on multiple antibiotic resistance of *E. fergusonii*, it could be inferred that antibiotic resistance among *E. fergusonii* is worrying as there could be possible transfer of genetic material among closely related species of *Enterobacteriaceae* and may pose a threat to treatment of common infections caused by bacteria.

Antibiotics are generally used to treat bacterial infections, however the prolonged use and misuse of antibiotics has led to the emergence of resistance strains among bacteria posing serious health concern. Of late, the threat posed by Extended-Spectrum Beta-Lactamase (ESBL) producing bacteria draws attention as beta-lactam antibiotics account for 60 % of the antibiotics used worldwide for the treatment of infectious diseases[32]. Beta-lactamases are enzymes that confer bacterial resistance to the beta-lactam family of antibiotics such as penicillins, cephalosporins, cephamycins, and carbapenems. These enzymes catalyze the hydrolysis of the amide bond of four-membered beta-lactam ring and render the antibiotic inactive[33]. The main β-lactamase producers belong to the family *Enterobacteriaceae*, *Escherichia coli* and *Klebsiella* in particular[17,34-36]. ESBL bla_{TEM} was detected in both the isolates of *E. fergusonii* and $bla_{CTX\text{-}M\text{-}1}$ in E060 in the current study (Table 4). However, bla_{SHV} could not be detected in both the *E. fergusonii* isolates. $bla_{CTX\text{-}M\text{-}1}$ reflects the potent hydrolytic activity of these β-lactamases against cefotaxime and they are not very closely related to bla_{TEM} or bla_{SHV} β-lactamases[17]. To date there are no reports on *E. fergusonii* from fish samples harbouring ESBL. A study on clinical *E. fergusonii* from Nigeria has confirmed the detection of bla_{TEM}, bla_{SHV} and $bla_{CTX\text{-}M\text{-}1}$ from three isolates of *E. fergusonii* and was the first to report detection of multiple beta-lactamase genes in carbapenem-resistant ESBL producing *E. fergusonii* from clinical samples[18]. In a study from *E. coli* isolated from urinary tract, of the three beta-lactamase (*bla*) genes, bla_{TEM} was detected among 38 (48.7 %), followed by $bla_{CTX\text{-}M\text{-}1}$ in six (7.6 %) and bla_{SHV} in four (5.1 %) phenotypically confirmed ESBL and non-ESBL isolates out of the total 78 isolates studied[37].

Initially AmpC-producing *Enterobacteriaceae* were found to be associated with hospitals and institutional care centres[38]; however, of late, this has been detected in food-producing animals[39], and retail meat[40], suggesting gene exchange between different reservoirs[41]. The two *E. fergusonii* isolates isolated in this study harboured AmpC gene. Study of these genes is important as they are responsible for producing different enzymes that hydrolyse all cephalosporins except cefepime and the carbapenems and confer resistance to penicillins, aztreonam, cephamycins, and narrow, broad and expanded-spectrum cephalosporins. AmpC are plasmid mediated ampicillinase and located chromosomally and can be transmitted by horizontal gene transfer[41]. AmpC prevalence has been reported in *Klebsiella* spp. (24.1 %) and *E. coli* (37.5 %) isolated from children under five in Chennai, India[43] and also from a referral hospital in Karnataka in which 3.3 per cent of isolates produced AmpC β-lactamases[44]. There are no studies found on AmpC in *E. fergusonii*.

Table 3 — Antibiotic resistance pattern of *E. fergusonii* isolates

S. No	Antibiotics	E011	E060
1	Ampicillin (30 µg)	R	R
2	Amikacin (30 µg)	S	S
3	Cefotaxime (30 µg)	R	R
4	Colistin (10 µg)	S	S
5	Chloramphenicol (30 µg)	S	R
6	Cefoxitin (30 µg)	S	R
7	Imipenen (10 µg)	S	S
8	Trimethoprim (5 µg)	R	R

S – Sensitive and R – Resistant

Table 4 — *E. fergusonii* harbouring antibiotic resistance genes

S. No	Isolate No.	bla_{TEM}	bla_{SHV}	$bla_{CTX\text{-}M\text{-}1}$	*AmpC*	*Sul1*	*Sul2*	*Integron*	bla_{NDM}
1	E011	+	-	-	+	-	-	-	-
2	E060	+	-	+	+	+	-	+	-

Three genes (*sul1*, *sul2* and *sul3*) have been identified encoding sulfonamide resistance[45,46]. *Sul1* has almost exclusively been found on large conjugative plasmids and on class 1 integrons. *Sul2* was previously considered to be located on small non-conjugative plasmids, but recently the gene has also been found on a wide range of large conjugative plasmids and have been linked to the prevalence of streptomycin resistance. *Sul3* was originally described from pigs in Switzerland in 2003 and has since been reported in animals as well as humans in many countries. *Sul1* was also detected in *E. fergusonii* isolate E060 in the present study.

NDM-1 was first identified in 2008 in a *Klebsiella pneumoniae* isolate recovered from a Swedish patient who has been previously hospitalized in New Delhi, India[47]. Subsequently, variants of *NDM* and $bla_{\text{NDM-5}}$ have been reported from fresh seafood in India[48]. However, *NDM* could not be detected in *E. fergusonii* isolates of present study.

The occurrence of drug resistant *E. fergusonii* in seafood is a public health concern as the bacteria could enter the food chain and threaten the effectiveness of the drugs. This is an emerging pathogen, therefore, further study has to be done to understand the pathogenicity of the bacterium and the risk factors associated with the consumption of seafood harbouring drug resistant bacteria.

Acknowledgements

The authors acknowledge Tamil Nadu Dr. J. Jayalalithaa Fisheries University for the support and facilities in carrying out this research work.

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical Statement

Not applicable.

Author Contributions

BC: Investigation, carried out the research and manuscript preparation; GS: Manuscript correction; SS: Formal analysis; and AAM: Investigation and formal analysis.

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