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Phenotypic variations among strains of *Escherichia coli* O157 isolated from raw milk samples collected in Kerala, South India

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ABSTRACT

Entero-haemorrhagic *Escherichia coli* (EHEC) constitutes a heterogeneous group of foodborne pathogens. We characterize 60 *E. coli* O157 serotypes isolated from 97 raw milk samples collected from local milk retailers. The biochemical analysis of the indigenous strains revealed a significant variation from their global counterparts. The study identifies a distinct phenotypic variant of *E. coli* O157 that ferments sorbitol, is urease-positive and produces hydrogen sulphide. The observed heterogeneity points to the inconsistency in the current screening approaches, which mostly depend on biochemical features, and justifies the demand for a universally inclusive standard procedure.

Keywords: Raw milk, EHEC; *E. coli* O157:H7; Shiga-toxigenic *Escherichia coli; STEC;* Sorbitol fermentation

INTRODUCTION

Enterohemorrhagic *Escherichia coli* (EHEC) typically causes acute bloody diarrhoea that can lead to haemolytic-uremic syndrome (HUS) and haemorrhagic colitis in 10% of the affected individuals (Fatima and Aziz., 2022). The prominent serotype of this class, Shiga-toxin producing *Escherichia coli* (STEC) O157:H7, is often associated with a few global outbreaks of Haemolytic Uremic Syndrome (HUS) (Vygen-Bonnet *et. al.*, 2017). The symptoms of STEC infection range from asymptomatic to mild abdominal cramps and diarrhoea to haemorrhagic colitis at a later stage (Marder *et al*., 2018). The infection, however, has sequelae of a fatal HUS (Vygen-Bonnet *et. al*., 2017).

The preliminary screening of STEC isolates and their variants is based on the fermentation pattern in Sorbitol-MacConkey (SMAC) medium enriched with cefixime-tellurite (Paton and Paton., 1998). Sorbitol fermentation is an important attribute that differentiates STEC O157 from non-O157 strains (Remis *et al.*, 1984., De Boer and Heuvelink., 2000, Smith *et. al.*, 2014). But, the sorbitol fermenting *E. coli* O157 (SF STEC) strains have been reported in several outbreaks (Karch *et. al*., 2001., Vygen-Bonnet *et. al*., 2017., Heiman *et. al*., 2015) across the globe. A notable example is SF STEC O157/H-, a strain implicated in an outbreak in Germany with fatal complications of HUS (Vygen-Bonnet *et. al*., 2017). Since the initial step in the screening and detecting STEC strains is still largely reliant on the sorbitol-fermentation (Feng *et. al*., 2002., O'Sullivan *et al.*, 2007) pattern, the chances of missing them in the screening procedure are high. This makes the procedure cumbersome and might interfere with the subsequent identification processes. Therefore, employing an effective strategy for detecting and discriminating sorbitol fermenting *E. coli* O157 strains is highly warranted. The findings corroborate with that of De Boer and Heuvelink, 2000.

MATERIALS AND METHODS

Characterization of the isolates Samples from multiple locations across the Indian state of Kerala were subjected to the study (Figure:1). Fresh raw milk samples (n=97) were collected from house-hold retailers of different districts of Kerala. The microbial analysis started within two hours of sample collection. Pre-enrichment for the screening of STEC was carried out in Novobiocin (2mg/100ml) supplemented EC Broth. 10 ml of milk was mixed with 90ml EC- Novobiocin broth and kept in a shaker-incubator for four hours. This was followed by immuno-magnetic bead mediated (Dynabead®, Applied Biosystems, Oslo, Norway) enrichment (O'Sullivan *et. al.*, 2007). Dynabead coated with antibodies against the surface antigen, O157, was

used (Joseph and Kalyanikutty, 2021). Further isolation was done by spreading the enriched broth onto Cefixime-Tellurite supplemented SMAC (HiMedia, Mumbai, India) agar.

i) Biochemical characterization

The biochemical parameters of the isolated STEC strains were evaluated using several tests specified in MacFaddin (2000), which included sugars such as glucose, galactose, lactose, sorbitol, amino acid; lysine decarboxylase, and urease and hydrogen sulphide production. IMViC tests were also performed following Harrigan and McCance's (1976) protocol. The

reference strain used for the study was CD101 (Genbank accession number MT912689), and the characteristics were compared with the standard strain EDL933 (Kwon *et. al*., 2017).

Figure 1: Geographical location of raw milk sample collected for the study. The blue coloured areas represent the districts where raw milk samples were collected.

ii) Serological Assay

The serological analysis was done using the LK-13 *E. coli* O157 latex agglutination kit (Himedia, Mumbai, India). The presence of O (somatic lipopolysaccharide) and H (flagellar) antigen on the surface (Nataro and Kaper., 1998) of the bacteria causes the agglutination reaction. The agglutination tests were performed according to the manufacturer's instructions.

iii) Genetic characterization of the strain

For further confirmation of the data, we isolated the genomic DNA using crude cell lysis method (Joseph and Kalyanikutty., 2020). The DNA was then amplified by PCR for 35 cycles using universal primers RWO1 (5'AACTGGAGGAAGGTGGGGAT3') and DG74 (5'AGGAGGTGATCCAACCGCA3') that flank the 370bp region of 16s rRNA (Matar *et. al*., 1998). The conditions for PCR involved denaturation at 95°C for 1min; annealing at 56°C for 1min; extension at 72°C for 1min and final extension at 72°C for 10min. The amplified DNA detected after electrophoresis in 0.8% gel was analyzed using gel documentation unit (Vilber-Lourmat, France).

The amplified fragments were sequenced using Applied Biosystems 3130 automated Genetic Analyzer (California, USA) in Agrigenome Labs, Kochi, Kerala. The nucleotide sequences were confirmed using NCBI-BLAST and were deposited in the

GenBank. Sequence alignments were done using MUSCLE and phylogenetic comparisons of the sequences for the examined sequences were performed using MEGA 11 software (Tamura *e*t. *al*., 2021).

RESULTS AND DISCUSSION

i) Characterization of *E. coli* O157 in unpasteurized milk samples

During the organoleptic evaluation, the raw milk samples collected for the analysis displayed good physical qualities such as aroma, colour, and homogeneity. Out of 97 raw milk samples collected, sixty harboured *E. coli* O157 isolates (Table 1). The study found that the *E. coli* strain in raw milk samples was at a prevalence rate of 26%, which is comparable with the earlier reports by Joseph and Kalyanikutty in 2021 (22% from Kerala) and Sehgal *et al*., in 2008 (1.8% from India). This variation observed could be attributed to the considerable difference in the climate and seasons, farm hygienic standards, and sample differences. On the other hand, the high prevalence of STEC in raw milk samples poses a public health danger and should be minimized by implementing suitable milk handling and processing techniques. This also validates the report by the CDC (CDC., 2007) on how milk and milk products might be a source of a high percentage of foodborne outbreaks worldwide.

Table 1: STEC strains isolated based on biochemical and serological analysis from the in raw milk samples.

Location (site number)	No: of milk samples	No: of isolates detected as *E. coli* on initial screening	No: of isolates confirmed as *E. coli* on biochemical screening	No: of STEC positive strains after serological analysis
Kasargod (1)	8	24	19	2
Kannur (2)	8	29	19	5
Kozhikode (3)	10	33	15	5
Palakkad (4)	8	23	19	4
Malappuram (5)	9	24	18	7
Thrissur (6)	10	28	19	8
Ernakulam (7)	9	30	17	6
Kottayam (8)	10	26	17	6
Alappuzha (9)	8	20	19	6
Kollam (10)	8	21	16	5
Thiruvanathapuram (11)	9	29	19	6
Total	97	287	230	60

ii) Biochemical screening

Further biochemical analysis of these isolates has revealed heterogeneity in the fermentation pattern, with 43(72%) of them exhibiting sorbitol fermentation (Figure 2). In addition, 15(25%) isolates produced urease and hydrogen sulphide (Figure 2), and the rest of the 17(28%) isolates displayed a non-sorbitol fermentation trait (Figure 2), the characteristics of a typical STEC O157:H7. The findings revealed that the indigenous *E. coli* O157 strains were biochemically diverse. A considerable proportion of the isolates were sorbitol fermenting. Though IMViC and other general biochemical tests that aid in the selective identification of STEC are consistent, fifteen isolates showed distinct traits for producing urease and hydrogen sulphide compared to those of their global counterparts. In their study on DNA banding patterns, heterogeneity among the STEC strains isolated from the same geographical region was recently reported by Joseph *et. al.* (2022). Sorbitol fermentation by *E. coli* O157 has been reported earlier (Karch *et. al.*, 2001, Vygen-Bonnet *et. al.*, 2017). However, the nature of sorbitol fermentation varies among strains reported from different parts of the world (Alpers *et.al.*, 2009, Pollock *et. al.*, 2010). The observation also calls into doubt the reliability of current screening methods in favour of more sensitive detection procedures.

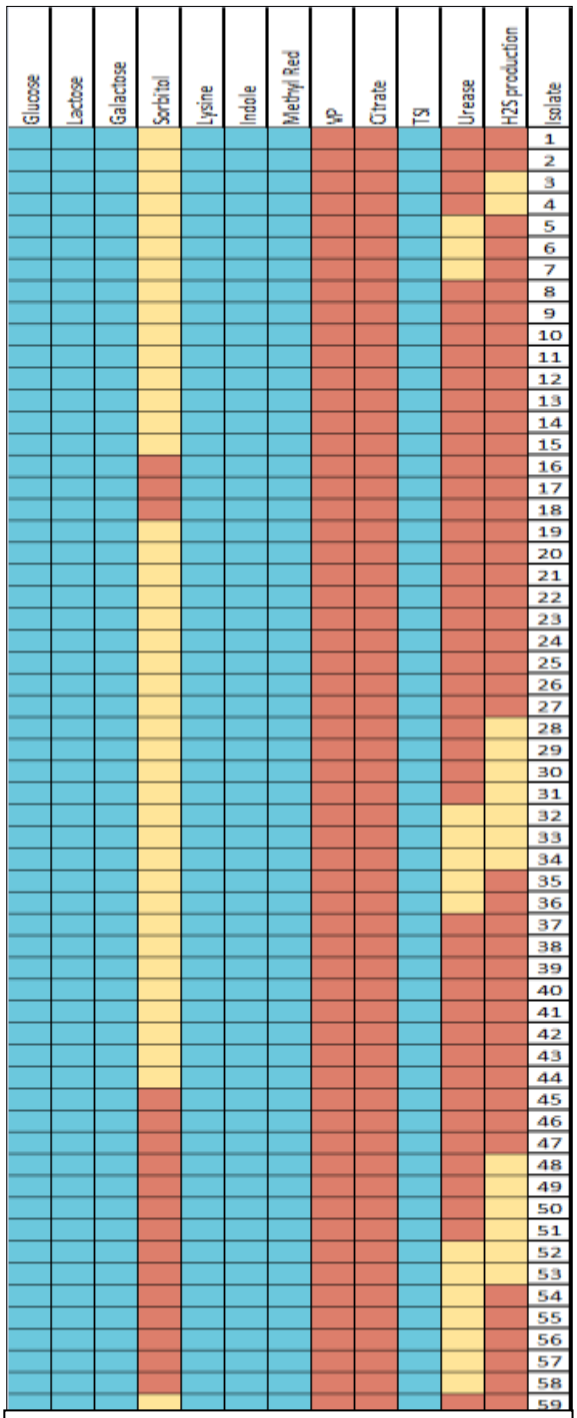

Figure 2: Heat map displaying the diversity of STEC isolates (n=60) in terms of its biochemical characteristics: Positive traits are denoted by blue colour, negative traits by red colour and the atypical phenotypes are represented by yellow colour yellow.

iii)Serotyping

STEC O157:H7 serotype, which is acknowledged as one of the major causative agents for serious foodborne outbreaks across the world (WHO, 2019). While unpasteurized milk samples from

Thrissur district (site no:7) had a larger number of EHEC O157:H7 strains (n=8), milk from Kasaragod district (site no:1) had the fewest (n=2) compared to milk from other districts (Table 1). However, it has not yet been determined whether the milk samples collected from the various sampling sites correlate with one another. To prevent the pathogen spread, milk collection centres and distribution facilities must have aseptic handling and quality control systems.

iv) Phylogenetic analysis

The isolates were partially sequenced, and a phylogenetic tree was constructed based on alignment with 16S rDNA genes retrieved from the NCBI database that showed high homology with the present sequences. The phylogenetic analysis demonstrated that the partial sequence of raw milk isolates obtained in the present study (ON970370.1) was clustered with *E. coli* O157:H7 isolated from a faecal sample of a patient in Texas, USA (CP089272.1,CP089032.1) and cattle feces sample in Louisiana, USA (CP040107.1) (Figure 2), indicating that the isolate might be genetically related to these two strains. The genetic relatedness among these strains could be due to the fact that they shared a common ancestor that might have transported via global trade and travel.

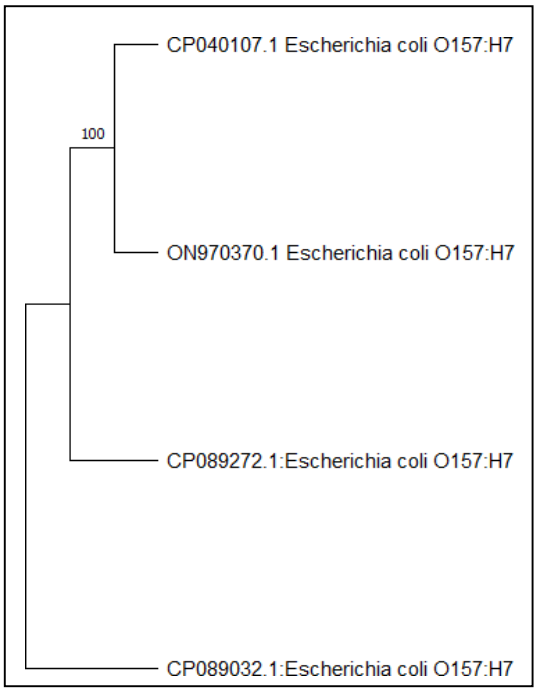

Figure 3: Phylogenetic analysis showing 100% genetic relatedness of ON970370.1 to its global counterpart CP040107.1 using MEGA 11 software with bootstrap values being confined to 500.

CONCLUSION

Analysis of STEC strains in raw milk samples collected from different geolocations of Kerala were indigenously diverse in terms of phenotypical characterization. The current approach for detecting *E. coli* O157 relies on preliminary screening of sorbitol non-fermenting colonies from sorbitol MacConkey agar plates. This could result in the omission of these pathotypes in the early stages of the detection procedure itself. Owing to the public health significance of EHEC, the present observations highlight the necessity of its comprehensive characterization and a respective restructuring of currently used isolation and detection methodologies.

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