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Are non-lactose-fermenting *Escherichia coli* important diarrhoeal pathogens in children and adults?

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Abstract:

Introduction. Diarrhoeagenic *Escherichia coli* (DEC) remains one of the major causes of acute diarrhoea episodes in developing countries. The percentage of acute diarrhoea cases caused by DEC is 30–40% in these countries. Approximately 10% of *E. coli* isolates obtained from stool specimens have been reported to be non-lactose-fermenting (NLF). The available literature is sparse regarding the pathogenicity of NLF *E. coli* causing infectious diarrhoea.

Aim. We aimed to elucidate the importance of NLF *E. coli* in causing diarrhoea in both adults and children by detecting various DEC pathotypes among NLF *E. coli* in stool samples taken from gastroenteritis cases.

Material and Methods. A total of 376 NLF *E. coli* isolates from 3110 stool samples from diarrhoea/gastroenteritis patients were included in the study. Up to three NLF colonies that were not confirmed as *Vibrio cholerae*, *Aeromonas* spp., *Salmonella* spp. or *Shigella* spp., but were identified as *E. coli* using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF), were carefully picked up from each MacConkey agar plate and then meticulously streaked onto freshly prepared, sterilized nutrient agar plates, and biochemical reactions were conducted. Multiplex PCR was conducted for the EAEC, EPEC, ETEC and EHEC pathotypes and PCR for the *ipaH* gene was conducted for EIEC. The disc diffusion method was used for antibiotic sensitivity testing.

Results. Using multiplex PCR and *ipaH* PCR, a total of 63 pathotypes of DEC were obtained, with EAEC being the most predominant (n=31) followed by EIEC (n=22), EPEC (n=8) and ETEC (n=2). To further differentiate EIEC from *Shigella*, additional biochemical tests were performed, including acetate utilization, mucate and salicin fermentation, and aesculin hydrolysis. Antimicrobial susceptibility testing (AST) showed that maximum resistance was seen against ciprofloxacin (82.5%) followed by ampicillin (77.8%) and cotrimoxazole (68.2%), and minimum resistance was seen against ertapenem (4.8%).

Conclusion. In our study two pathotypes (EAEC, EIEC) were predominant among NLF *E. coli* and these were not only important aetiological agents in children, but also in adults. Our study also sheds light on the epidemiology of EIEC, which is one of the most neglected DEC pathotypes, as hardly any microbiological laboratories process NLF *E. coli* for EIEC.

DATA SUMMARY

All supporting data is provided in the manuscript.

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Keywords: adults; diarrhoea; *Escherichia coli*; enteroinvasive *Escherichia coli*; non-lactose-fermenting.
Abbreviations: APW, alkaline peptone water; DEC, diarrheagenic *Escherichia coli*; EAEC, enteraggregative *Escherichia coli*; *E. coli*, *Escherichia coli*; EHEC, enterohemorrhagic *Escherichia coli*; EIEC, enteroinvasive *Escherichia coli*; EPEC, enteripathogenic *Escherichia coli*; ETEC, enterotoxigenic *Escherichia coli*; *ipa*, invasion plasmid antigen; NLF, non-lactose fermenting; ONPG, o-nitrophenyl-β-D-galactopyranoside; TCBS, thiosulfate-citrate-bile salts-sucrose agar; XLD agar, xylose lysine deoxycholate agar.
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INTRODUCTION

Infectious diarrhoea plays an important role in morbidity and mortality worldwide, especially in children under 5 years old in the developing world [1–3]. Among the bacterial causes, diarrhoeagenic *Escherichia coli* (DEC) pathotypes, which cause a range of illnesses varying in severity from acute watery diarrhoea to dysentery, are the pivotal aetiological agents [4]. Based on their virulence and phenotypic characteristics, DEC are categorized into six main pathotypes, i.e. enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), Shiga toxin-producing *E. coli* (STEC) and diffusely adherent *E. coli* (DAEC).

Up to 10% of isolates of *E. coli* have historically been stated to be slow or non-lactose fermenting (NLF), although the clinical implications of isolating these are unknown [5]. In most routine microbiology laboratories, only lactose-fermenting colonies of *E. coli* are further evaluated for DEC. NLF colonies, after *Shigella* and *Salmonella* are excluded, are not processed further for DEC pathotypes. Although a few studies have tried to elucidate the pathogenicity of NLF *E. coli,* the available literature are sparse regarding the pathogenicity of NLF *E. coli* causing infectious diarrhoea.

An important organism to look for will be enteroinvasive *E. coli* (EIEC), which tends to be invasive in colonic epithelial cells, where it causes an illness that is very similar to shigellosis. Biochemically, EIEC is also similar to *Shigella*, and very few biochemical tests can distinguish EIEC from *Shigella*. Therefore, in many cases the EIEC pathotype is misreported as *Shigella* [6]. EIEC also shares invasion plasmids with *Shigella*, hence molecular methods based on *ipaH* antigens are used for its detection. There are hardly any data available on the epidemiology of EIEC in the literature. We aimed to elucidate the importance of NLF *E. coli* in causing diarrhoea in both adults and children by detecting various DEC pathotypes (EIEC, EPEC, ETEC, EAEC, EHEC) among NLF *E. coli* obtained in stool samples from patients with gastroenteritis, and found that two DEC pathotypes, i.e. EIEC and EAEC, will be missed in a considerable proportion of stool specimens if NLF *E. coli* are not evaluated for pathogenicity.

METHODS

Sample selection

A total of 376 NLF *E. coli* isolates were then isolated from 3110 stool samples from gastroenteritis or diarrhoea patients from the out-patient department (OPD) and the in-patient department (IPD), covering all age groups from different wards of PGIMER, Chandigarh from January 2019 to April 2021 (Fig. 1). Clinical data for the diarrhoeal episodes and other related symptoms were collected for each case. It was mandatory for each patient to provide written, informed consent.

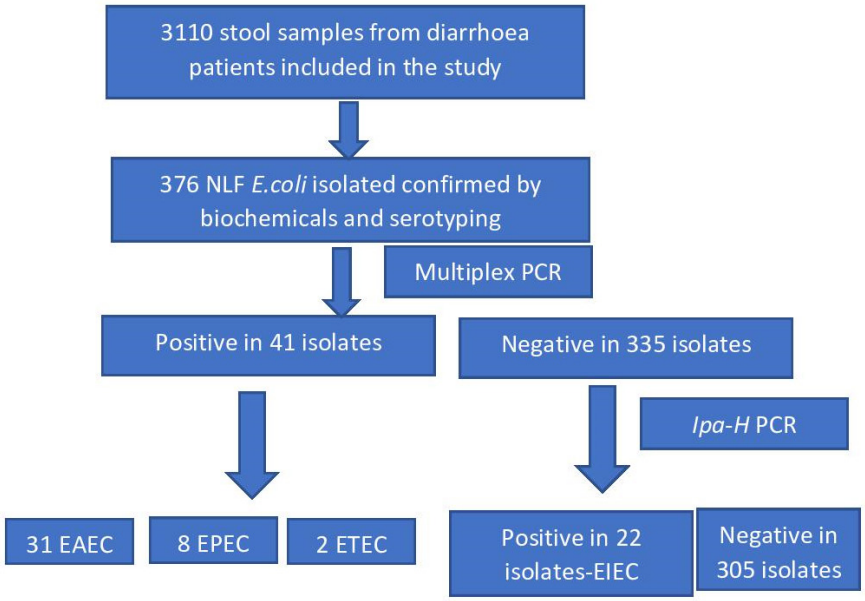

Fig. 1. Flow chart showing the scheme of the study.

Sample collection and processing

Stool samples from the gastroenteritis or diarrhoea patients were collected in sterile containers, inoculated into a transport medium, Cary Blair medium, and sent to the laboratory via cold chain. They were cultured for the presence of *Aeromonas* sp., *Vibrio cholerae*, *Salmonella* spp., *Shigella* spp. and *E. coli* using standard procedures [7]. Samples were then inoculated onto selenite F broth, MacConkey agar, xylose lysine deoxycholate (XLD) agar, ampicillin blood agar, thiosulfate–citrate–bile salts–sucrose agar (TCBS) agar and alkaline peptone water (APW) and then incubated at 37 °C for 18–24 h. The organisms found were then identified using standard biochemicals [7] and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF), which was performed on a MALDI Microflex LT mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany). Up to three NLF colonies that were not confirmed as *Aeromonas* spp., *V. cholerae*, *Salmonella* spp., or *Shigella* spp., but were identified as *E. coli* on MALDI-TOF, were selected from each MacConkey agar plate and streaked onto fresh, sterilized nutrient agar and stored in trypticase soy broth containing 15% glycerol at −80 °C.

Biochemical reactions

Biochemical reactions were conducted for all the putative NLF *E. coli* that were negative for serotyping (Denka Seiken Co. Ltd, Japan) of *Shigella* to differentiate them from *Shigella* species [8]. These included triple sugar iron, motility, indole test, lysine decarboxylase, citrate utilization, glucose and xylose fermentation with Durham's tube, mannitol fermentation and o-nitrophenyl-β-D-galactopyranoside (ONPG) test (Fig. 2).

Extraction of DNA from confirmed *E. coli*

DNA from each confirmed NLF *E. coli* isolate was extracted via the heat extraction method as described by Dilhari *et al.* [9]

Identification of DEC genes

A multiplex PCR for enterotoxins [heat-labile (LT) and heat-stable (ST)], EPEC (Eae) protein bundle-forming protein (Bfp), Shiga toxins (Stx1, Stx2), VTcom for EHEC and (CVD432) for EAEC was performed using published primers [9–14] and the protocol shown in Table 1.

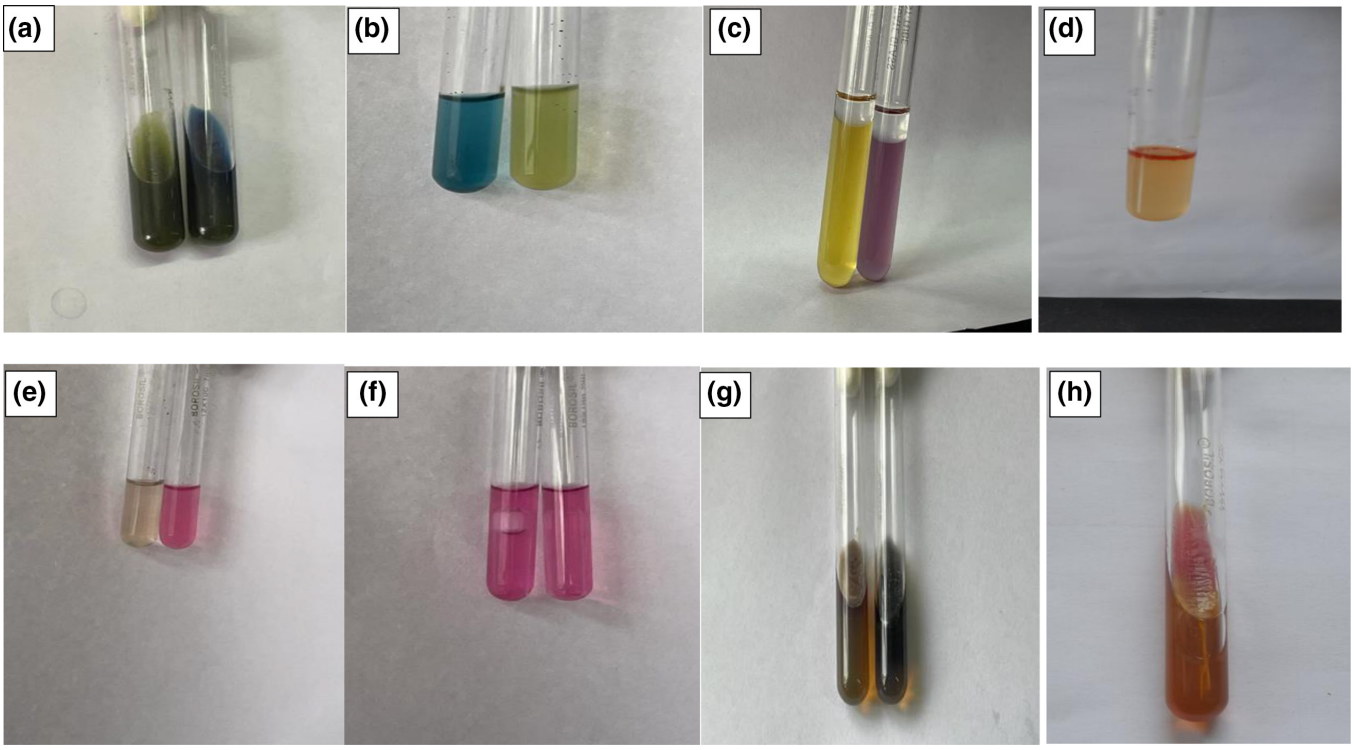

Fig. 2. Biochemical reactions for EIEC. (a) Acetate utilization test (green, negative; blue, positive). (b) Mucate fermentation (blue, positive; yellow, negative). (c) Lysine derboxylase (yellow, control; purple, positive). (d) Positive indole test. (e) Salicin fermentation (colourless, negative; pink, positive). (f) Glucose fermentation with gas/without gas. (g) Bile aesculin hydrolysis (yellow, negative; black, positive). (h) Triple sugar iron (K/A with gas formation).

Table 1. List of primers, their sequences and the size of the amplified products used in this study

Target gene	Primer sequence (5'–3')	Primer designation	PCR product size (bp)	DEC pathotype	Reference
Bfp	F: GGAAGTCAAATTCATGGGGGTAT R: GGAATCAGACGCAGACTGGTAGT	Bfp	300	EPEC	[10]
Eae	F: TCAATGCAGTTCCGTTATCAGTT R: GTAAAGTCCGTTACCCCAACCTG	Eae	482	EPEC	[10]
Elt	F: ACGGCGTTACTATCCTCTC R: TGGTCTCGGTCAGATATGTG	LT	273	ETEC	[11]
CVD432	F: CTGGCGAAAGACTGTATCAT R: AATGTATAGAAATCCGCTGTT	pCVD432	630	EAEC	[12]
estA1	F: TCTTTCCCCTCTTTTAGTCAG R: ACAGGCAGGATTACAACAAAG	STp	166	ETEC	[11]
estA2-4	F: TTCACCTTTCCCTCAGGATG R: CTATTCATGCTTTCAGGACCA	STh	120	ETEC	[11]
stx1	F: CAGTTAATGTGGTGGCGAAGG R: CACCAGACAATGTAACCGCTG	Stx1	348	EHEC	[10]
stx2	F: ATCCTATTCCCGGGAGTTTACG R: GCGTCATCGTATACACAGGAGC	Stx2	584	EHEC	[10]
stx1 +stx2	F: GAGCGAAATAATTTATATGTG R: TGATGATGGCAATTCAGTAT	VTcom	518	ETEC	[13]
ipaH	F: GAAAACCCTCCTGGTCCATCAGG R:GCCGGTCAGCCACCCTCTGAGAGTAC	*ipaH*	437	EIEC	[14]

F, forward primer; R, reverse primer.

PCR conditions for DEC genes

In multiplex PCR reactions two or more pairs of primers were used. All the PCR reactions were performed in 20 µl final volume containing 0.5 µl of the template DNA, 1 µl of DNA polymerase, 0.2 mM of each dNTP, 1.5 mM of $MgCl_2$ and 10 µM of each primer. The thermocycling conditions for all the PCRs were as follows: 95 °C for 2 min, 95 °C for 15 s, 52 °C for 8 s and 10 s at 72 °C for 30 cycles, with a final 2 min extension at 72 °C. PCR products were subsequently analysed on a 1.5% agarose gel electrophoresis in Tris–borate–EDTA with ethidium bromide (EtBr) staining.

Defining criteria for EIEC

NLF *E. coli* isolates negative by the above DEC multiplex PCR were subsequently tested for identification of EIEC using the *ipaH* gene (Table 1).

PCR for *ipaH* gene

In a single PCR reaction, one pair of primers was used. PCR reaction was conducted at a final volume of 25 µl comprising 0.5 µl DNA, 1 µl DNA polymerase, 2.6 mM of each dNTP, 1.5 mM of $MgCl_2$. Primer was used at 10 µM concentration [15].

The thermocycling parameters for PCR (*ipaH* gene)

The thermocycling parameters for the *ipaH* gene in PCR were as follows in the thermal cycling programme: initial denaturation at 95 °C for 5 min, 1 cycle; denaturation at 94 °C for 30 s, 35 cycles; annealing at 58 °C for 30 s, 35 cycles; extension at 72 °C for 1 min, 35 cycles; final extension at 72 °C for 7 min, 1 cycle; and holding at 4 °C, 1 cycle. The amplification products were electrophoresed through a 2% agarose gel and visualized with UV transilluminator after EtBr staining. A 100 bp DNA ladder was used as a molecular size marker in the gel.

Antibiotic sensitivity testing

Antibiotic sensitivity testing was performed with the disc diffusion method for the following antibiotics: cefepime (15 mg), piperacillin–tazobactam (10 mg), ertapenem (10 mg), ampicillin (10 mg), ciprofloxacin (5 mg), gentamicin (10 mg), cotrimoxazole (25 mg), cefoxitin (30 mg), amikacin (30 mg), imipenem (10 mg), levofloxacin (5 mg) and ceftriaxone (30 mg) as per the Clinical

Table 2. Biochemical reactions of NLF *E. coli* included in the study

Isolates	TSI	Indole		ONPG		Motility		Lysine decarboxylase		Citrate utilization		Glucose fermentation with gas	Mannitol fermentation		Xylose fermentation with gas
		+	–	+	–	Motile	Non-motile	+	–	+	_		+	–	
NLF *E. coli* (376)	K/A with gas	376	0	374	2	358	18	376	0	0	376	376	374	2	376

and Laboratory Standards Institute (CLSI) guidelines [16]. Multidrug resistance was described as acquired resistance to three or more antimicrobials from different antimicrobial classes tested.

Serotyping for *E. coli* O serotypes

All EAEC and EIEC isolates were sent to the National *Salmonella* and *Escherichia* Centre, Central Research Institute, Kasauli (HP), India for *E. coli* O serotyping [17].

RESULTS

Out of a total of 3110 stool samples, 376 isolates of NLF *E. coli* were obtained from 376 patients suffering from gastroenteritis with an average age of 31±2 years. Out of 376 patients, 199 were male and 177 were female, while 180 patients were from the OPD and 196 were from the IPD. Biochemical reactions, along with serotyping for *Shigella*, were conducted for all 376 isolates to differentiate them from *Shigella* species (Table 2, Fig. 2). All of the isolates were negative for indole production, on triple sugar iron agar all the isolates showed K/A reaction with gas production, lysine production was positive for all the isolates and out of all 4.8% (18) were non-motile. Using multiplex PCR (Fig. 3) and *ipaH* PCR (Fig. 4), a total of 63 pathotypes of DEC were obtained,

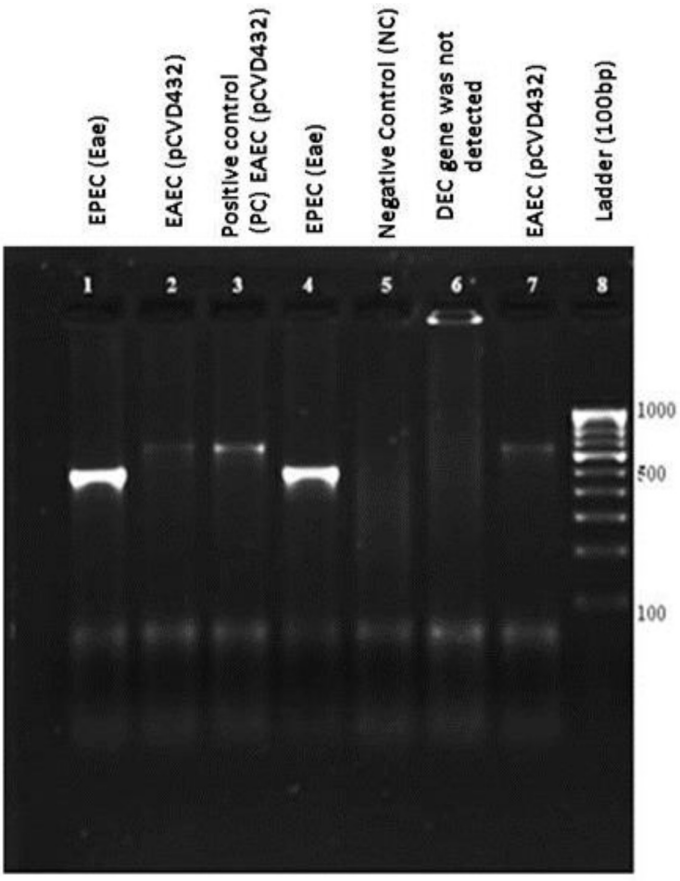

Fig. 3. A representative gel electrophoresis profile of DEC genes. Lane 1, EPEC (*eae*); lane 2, EAEC (*pCVD432*); lane 3, positive control (PC); lane 4, EPEC (*eae*); lane 5, negative contol (NC); lane 6, DEC gene was not detected; lane 7, EAEC (*pCVD432*), lane 8, ladder (100 bp).

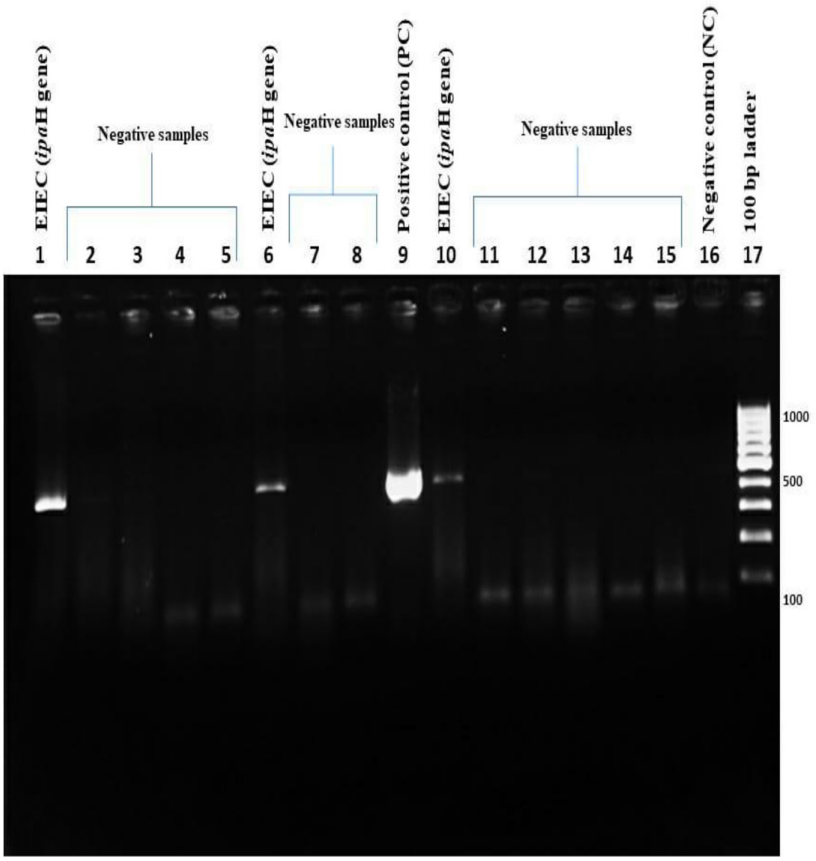

Fig. 4. A representative gel electrophoresis profile of the *ipa*H gene. Lane 1, EIEC (*ipa*H); lanes 2, 3, 4, 5, *ipa*H gene was not detected; lane 6, EIEC ; lane 7, 8, *ipa*H gene was not detected; lane 9, positive control (PC); lane 10, EIEC (*ipa*H); lanes 11, 12, 13, 14, 15, *ipa*H gene was not detected; lane 16, negative control (NC); lane 17, ladder (100 bp).

with EAAC being the most predominant (*n*=31) followed by EIEC (*n*=22), EPEC (*n*=8) and ETEC (*n*=2). Details of demographic profiles and clinical presentation of cases from which DEC were isolated are given in Table 3. The overall average age from the samples yielding DEC in our study was 31±2 years, which was similar for EAEC (31.6 years) and ETEC (30.4 years). The average age for EIEC was 20.7 years and for EPEC it was 11 years. A total of 57% (36/63) patients have been hospitalized.

The biochemical profiles of these DEC pathotypes are shown in Table 4. To further differentiate EIEC from *Shigella*, additional biochemicals were performed, including fermentation of mucate, acetate utilization, and salicin fermentation and aesculin hydrolysis (Table 5). DEC were serotyped to determine the O antigens of EIEC and EAEC. Out of a total of 22 isolates of EIEC, *E. coli* O serotypes were detected in 15 isolates, and 7 strains were untypeable. The most common serotype was O120, present in four isolates, followed by O98 and O7, each in three isolates. Among 31 EAEC isolates, the most commonly encountered serotypes were O22, O88 and O149 (*n*=4), followed by O17 (*n*=3) and O11 (*n*=3); 2 isolates were of the O35 serotype, 2 were O84, and a single isolate each were O126, O128, O49 and O8 (Table 5). Antimicrobial susceptibility testing (AST) showed that maximum

Table 3. Demographic profile and clinical profile of diarrhoeagenic *E. coli* (DEC)

Clinical characteristics	Average age (years)	Sex		Opd/IPD		Diarrhoea		Diarrhoea with dysentery *n* (%)	Fever *n* (%)	Abdominal pain *n* (%)
		Male *n* (%)	Female *n* (%)	OPD *n* (%)	IPD *n* (%)	Acute *n* (%)	Chronic/persistent *n* (%)			
EAEC (31)	31.6	19 (61.2%)	12 (38.7%)	µ (32.2%)	21 (67%)	12 (38.7%)	18 (58%)	3 (9%)	4 (12.9%)	10 (32.2%)
EIEC (22)	20.7	15 (68.2%)	7 (31.8%)	10 (45.5%)	12 (54.5%)	10 (45.5%)	12 (54.5%)	9 (40.1%)	17 (77.3%)	20 (90.9%)
EPEC (8)	11	5 (62.5%)	3 (37.5%)	4 (50%)	2 (25%)	5 (62.5%)	3 (37.5%)	0	5 (62.5%)	6 (75%)
ETEC (2)	30.4	1 (50%)	1 (50%)	1 (50%)	1 (50%)	2 (100%)	0	0	1 (50%)	1 (50%)

Table 4. Biochemical reactions of DEC pathotypes

DEC (64)	TSI	Indole		ONPG		Motility		Ornithine decarboxylase		Lysine decar -boxylase		Arginine dihydrolase		Citrate utilization	Glucose fermentation with gas	Mannitol fermentation	
		+	−	+	−	Motile	Non-motile	+	−	+	−	+	−			+	−
ETEC (2)	K/A with gas	2	0	2	0	2	0	1	1	2	0	0	2	−	+	2	0
EPEC (8)	K/A with gas	8	0	8	0	8	0	3	20	8	0	3	5	−	+	8	0
EAEC (31)	K/A with gas	31	0	31	0	28	3	10	13	31	0	15	16	−	+	31	0
EIEC (22)	K/A with gas	22	0	21	2	4	18	10	12	22	0	6	16	−	+	21	1

Table 5. *Escherichia coli* O serotyping and biochemicals for EIEC

Serotype	Mucate fermentation		Acetate utilization		Salicin fermentation		Esculin hydrolysis	
	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
O120 (*n*=4)	3	1	4	0	4	0	4	0
O98 (*n*=3)	3	0	3	0	3	0	3	0
O7 (*n*=3)	1	2	3	0	3	0	2	1
O26 (*n*=1)	1	0	1	0	0	1	1	0
O84 (*n*=1)	1	0	1	0	1	0	1	0
O86 (*n*=1)	0	1	1	0	1	0	1	0
O105 (*n*=1)	1	0	1	0	1	0	1	0
O18 (*n*=1)	1	0	1	0	1	0	1	0
Untypeable (*n*=7)	5	2	7	0	6	1	4	3

resistance was seen against ciprofloxacin (82.5%) followed by ampicillin (77.8%) and cotrimoxazole (68.2%), while minimum resistance was seen against ertapenem (4.8%). Among the different DEC pathotypes, significant numbers were only obtained for EAEC (31) and EIEC (22) to comment on the resistance pattern. Among EAEC, maximum resistance was seen against ampicillin (87.1%) and ciprofloxacin (87.1%), followed by ceftriaxone (74.2%) and cotrimoxazole (64.5%). Among EIEC strains, maximum resistance was seen against ciprofloxacin (81.8%) and cotrimoxazole (81.8%), followed by ampicillin (72.7%). Statistical analysis was only performed between EAEC and EIEC by using Fischer's exact test. Statistical analysis was performed using GraphPad Prism v.6.0 (GraphPad Software, La Jolla, CA, USA) and a P-value <0.05 was taken as significant. The antimicrobial resistance pattern of DEC is presented in Table 6.

DISCUSSION

Among children <5 years old in developing countries, diarrhoeagenic *Escherichia coli* (DEC) are responsible for ~30–40% of acute diarrhoea episodes [18], and are also an important cause of both sporadic cases and diarrhoeal outbreaks all over the world [19]. DEC contain and express various virulence factors that enable them to exhibit pathogenicity. NLF colonies are usually processed for *Salmonella*, *Shigella* and *Vibrio cholerae*. Although NLF *E. coli* are commonly isolated, they are usually not processed further. Moreover, *Shigella* are especially difficult to differentiate from NLF *E. coli* due to similar colony characters. MALDI-TOF also cannot differentiate between these two species. Therefore, reliance is placed on biochemicals such as indole, ONPG, lysine decarboxylase, acetate utilization and mucate fermentation and agglutination by antisera. *Shigella* strains are always lysine decarboxylation-negative, produce indole variably, are acetate utilization- and mucate fermentation-negative and

Table 6. Antimicrobial resistance in DEC pathotypes

Statistically significant (*P*<0.05) when antibiotic resistance among EAEC and EIEC was compared.

Antibiotic	Total, *n*=63 (%)	ETEC, *n*=2 (%)	EPEC, *n*=8 (%)	EAEC, *n*=31 (%)	EIEC, *n*=22 (%)	*P*-value
Ampicillin	49 (77.8%)	1 (50%)	5 (62.5%)	27 (87.1%)	16 (72.7%)	0.29
Ciprofloxacin	52 (82.5%)	1 (50%)	6 (75%)	27 (87.1%)	18 (81.8%)	0.70
Cefixime	16 (25.4%)	0	3 (37.5%)	3 (9.7%)	10 (45.5%)	**0.004***
Cefoxitin	17 (27%)	0	2 (25%)	6 (19.4%)	9 (40.9%)	0.12
Ceftriaxone	41 (65.1%)	1 (50%)	4 (50%)	23 (74.2%)	13 (59%)	0.37
Amikacin	9 (14.3%)	0	2 (25%)	4 (12.9%)	3 (13.6%)	1.0
Gentamicin	16 (25.4%)	0	2 (25%)	10 (32.2%)	4 (18.2%)	0.34
Imipenem	9 (14.3%)	0	1 (12.5%)	5 (16.1%)	3 (13.6%)	1.0
Ertapenem	3 (4.8%)	0	1 (12.5%)	1 (3.2%)	1 (4.5%)	1.0
Levofloxacin	27 (42.9%)	1 (50%)	2 (25%)	12 (38.7%)	12 (54.5%)	0.28
Piperacillin–tazobactam	10 (15.9%)	0	1 (12.5%)	6 (19.3%)	3 (13.6%)	0.72
Cotrimoxazole	43 (68.2%)	1(50%)	4 (50%)	20 (64.5%)	18 (81.8%)	0.22

ONPG-negative, except 15% cases of *Shigella dysentriae* type 1, all *Shigella sonnei* and 8% of *Shigella boydii* [8]. There is very little in the literature regarding the pathogenicity of NLF *E. coli*, as most of the studies focus on lactose-fermenting *E. coli*. Diarrhoeal disease data across the developing world focus on the paediatric population and very few studies from India have included adults [3, 20, 21]. Hence, to fill this gap the present study assessed the importance of NLF *E. coli* in stool specimens in diarrhoea cases across all age groups.

In the present study, 376 (12%) NLF *E. coli* were obtained from 3110 stool samples, which yielded a total of 63 (16.7%) strains of DEC. Similar to our study, Hossaine *et al.* [22] studied 74 (16%) NLF *E. coli* in 460 diarrhoeal stools from children <5 years of age in Bangladesh and found 24 (32%) strains of DEC. In our study, EAEC and EIEC were the predominant pathotypes in contrast to the study by Hossaine *et al.,* where EPEC followed by EAEC were the most common [22]. This difference could be because of the different age groups included in both of the studies. The average age of patients from whom DEC were isolated was 31.6 years for EAEC and 20.7 for EIEC. Our study highlights the role of EAEC and EIEC as a potential cause of diarrhoea not only in paediatric patients but also in adult patients. Moreover, both these pathotypes caused severe illness requiring hospitalization (67 % in EAEC and 52 % in EIEC).

Extensive geographical variations are observed in the prevalence of EAEC across the world and even in different regions of India [22–24]. Most of the previous studies on EAEC have only included paediatric patients [22, 25] and there is much less in the literature regarding the role of EAEC in the adult population [25–27]. In our previous study on lactose-fermenting *E. coli*, EAEC was present in a higher proportion (11.4%), followed by EPEC (6.2%) and ETEC (4.9%), but these samples were not processed for EIEC and only paediatric patients (<10 years age) were included [28]. A recent study by Jensen *et al.* investigated the prevalence of EAEC in adult Danish patients suffering from diarrhoea and healthy controls, concluding that asymptomatic carriage of EAEC is not common in the Danish adult patients (1.2%) as compared to children (10.5%), whereas in symptomatic diarrhoeal patients, EAEC was detected in 4.6% of patients and the median age was 34 years [26]. Similarly, in another study from Brazil, an increasing trend of isolation of EAEC in diarrhoeal stools in the adult population (57.9%) as compared to children (42.1%) was observed [27]. An interesting clinical presentation in our study was that 9.7% of patients presented with dysentery and 56% of EAEC cases presented with chronic/persistent diarrhoea. Jensen *et al.* in their study found that 39.7% of EAEC patients had chronic diarrhoea and 15 % had bloody diarrhoea, similar to our study [26]. EAEC is known to cause intestinal inflammation, as evidenced by several studies in which the presence of faecal lactoferrin and proinflammatory cytokines, notably interleukin (IL)−8, was observed [29–35]. A growing number of studies have supported the association of EAEC with persistent/chronic diarrhoea in malnourished children and individuals with HIV infection in developing countries [36–38]. However, in our study, although 18 patients presented with persistent diarrhoea, none of them were immunocompromised and 10 of them were adult patients.

The next most common DEC pathotype in our study was EIEC, which shares an invasion plasmid antigen with *Shigella* [39, 40]. Based on the *ipaH* gene expression alone, it is difficult to differentiate between *Shigella* and EIEC [39]. The few

biochemical properties that enable differentiation of *E. coli* and *Shigella* spp. are acetate utilization and mucate fermentation. EIEC may be positive for one or both of the properties, while *Shigella* strains are negative for both [8]. In our study all of the EIEC strains(n=22) were positive for either one (n=6) or both (n=16) of them. In addition, EIEC share the ability to produce gas from glucose and fermentation of xylose with other *E. coli* pathotypes . Salicin fermentation and aesculin hydrolysis may also be useful to differentiate between *Shigella* and EIEC [41]. *Shigella* is unable to ferment salicin and hydrolyze aesculin. In our study, 91% of EIEC strains were positive for salicin fermentation and 82 % hydrolyzed aesculin.

The clinical illness caused by EIEC resembles *Shigella* and is characterized by abdominal cramps, diarrhoea, vomiting, fever, chills and a generalized malaise [39]. In our study only 39% of EIEC patients presented with dysentery and 74% of patients presented with other features of invasiveness, such as fever and abdominal cramps. A highlight of our study was the demonstration of EIEC causing chronic diarrhoea (56%). We described for the first time such a presentation associated with EIEC infection. Usually, EIEC are only suspected in stool samples with mucus and blood, and not in other diarrhoeal cases, suggesting that the actual prevalence of EIEC may be higher than has been indicated in previous studies [39, 42].

Antibiotics may be required to treat invasive infections and persistent/chronic diarrhoea. Similar to other studies from India and Bangladesh, DEC pathotypes exhibited alarming rates of resistance against widely used antimicrobials – ciprofloxacin (82.5%), ampicillin (77.8%) and cotrimoxazole (68.2%) – and minimum resistance was seen against ertapenem (4.8%) [43–46]. Among the different DEC pathotypes, significant numbers were only obtained for EAEC (31) and EIEC (22) to make a comparison. The difference in the resistance pattern among EAEC and EIEC strains was not statistically significant, except for cefixime, as 45.5% of EIEC isolates were resistant to cefixime as compared to 9.7 % EAEC isolates, (P-value=0.004). Very few studies to date have reported antibiotic resistance in EIEC strains [46, 47]. Chellapandi *et al.* have also reported high rates of resistance in their EIEC isolates among the northeast Indian population [47]. This resistance is concerning and needs to be monitored at the community level.

CONCLUSION

This study is to the best of our knowledge the first one to highlight the importance of NLF *E. coli* in the aetiology of diarrhoea and gastroenteritis. In our study two pathotypes (EAEC, EIEC) were predominant among NLF *E. coli* and these are not only important aetiological agents in children, but also in adults. Our study also sheds light on the epidemiology of EIEC, which is the most neglected pathotype, as hardly any microbiological laboratories process NLF *E. coli* for EIEC. Moreover, in our study 56% of patients were admitted, highlighting the importance of severity of illness caused by NLF *E. coli*.

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Author contribution
Conceptualization: N.T., B.S., V.M. Investigation: B.S.,V.M, J.M., C.N., A.K., V.K., R.V. Supervision: N.T., B.M. Visualization: N.T., B.M. Writing – original draft: B.S., V.M., A.K., J.M., C.N. Writing – review and editing: N.T., B.M.

Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
The study was approved by the Postgraduate Institute of Medical Education and Research (PGIMER) Ethics Committee with reference no. INT/IEC/2017/173.

Consent to publish
Written informed consent for publication of their clinical details was obtained from the patients or the parents/guardians/relatives of the patients.

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Peer review history

VERSION 2

Editor recommendation and comments

https://doi.org/10.1099/acmi.0.000459.v2.3
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Georgios Efthimiou; University of Hull, Biomedical Science, Hardy Building, Cottingham Road, UNITED KINGDOM, Hull

Date report received: 02 January 2023
Recommendation: Accept

Comments: The work presented is clear and the arguments well formed. This study would be a valuable contribution to the existing literature. This is a study that would be of interest to the field and community. All comments by the reviewers were satisfactorily addressed.

SciScore report

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iThenticate report

https://doi.org/10.1099/acmi.0.000459.v2.2
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Author response to reviewers to Version 1

S.No.	Reviewer's comment	Reply by Author
Reviewer 1		
1	Figures 2 and 3 need modification. An appropriate legend to describe each multiplex would help the reader understand more clearly what is being presented. Also samples on gel images should not be labelled as "negative samples". They should be given a name and stated not detected in the legend. Negative sample may just indicate the assay was not sensitive enough to detect the target of interest.	A Legend has been added and corrections have been made.
2	L133- Reference how these methods are performed	Referred to line 136 as 8

3	Section 149- PCR conditions for DEC genes. There appears to be an initial PCR mix used followed by a final PCR reaction which contains difference concentrations of MGcl2 and primers. Please clarify which should be used in each instance.	We have made corrections Line no.148-155
4	L152 - "1.5 mM of MgCl2, at a final concentration of 1.5mM"- this is confusing, it is sufficient to state a final concentration.	Correction has been done. Line no.151
5	L154-10 µM concentration should be 10 µM concentration	Corrected line number L-151
6	L154- were as follows. Should be were as follows;	Corrected line number L-152
7	L157- Amplified samples were tested for 1.5% agarose gel electrophoresis in Tris-borate-EDTA and EtBr staining. Perhaps consider PCR products were subsequently analysed on a 1.5% agarose gel electrophoresis in Tris-borate-EDTA and EtBr staining	Corrected Line number 153-154
8	L160- "were future subjected to" please consider were subsequently tested for	Corrected Line number 157
9	L161- remove reference 14 ad it is provided in Table 1	Reference was removed from the table and added in text only
10	Section 162 PCR for ipaH gene- same comment as for section 149: There appears to be an initial PCR mix used followed by a final PCR reaction which contains difference concentrations of MGcl2 and primers. Please clarify which should be used in each instance	Correction has been done Line number160-162
11	L169 would suggest formatting PCR conditions in same format as lines 155-156	Corrected 160-162
12	L182-183- states "All EAEC and EIEC isolates were sent to National Salmonella and Escherichia Centre, Central Research Institute, Kasauli (HP), India for Escherichia coli O serotyping". Please reference the method used for serotyping	A reference no.17 has been added.

13	Line 206 - "most common serotype was O22, O88, O149 (4 in number each)" suggest reword to most common serotype was O22, O88, O149 (n=4)	Correction has been made from Line no.202-204
14	Line 207- (3) suggest change to (n=3)	Corrected line number 204
15	Line 217- Please provide a reference to Fischers test	Added Line no.213-215
16	L225- "Though NLF E.coli is commonly isolated, they are usually not processed further" - please provide supporting references for this statement. Also place a space between E. and coli	This is a common laboratory practice which we have seen being followed in many laboratories of India incuding our laboratory which is a referral laboratory for many centres.
17	L229-231 please provide references for % figures used	See reference 8
18	L237- In the present study,376 please insert space between comma and 376	Corrected line number 234
19	L255- et al change to et al.	Corrected line number 251
20	L270 "but none of them was immunocompromised" change to but none of them were immunocompromised	Corrected line number266
21	Throughout text of manuscript Tables and tables are used. Please change table to Table throughout e.g L200, 218 etc.	All lines were corrected
22	Table 1: State which is forward, and which is reverse primer	Corrected
23	Table 1: Please state if primers are in 5' to 3' orientation	Corrected
Reviewer 2		
24	1) Based on the amount of tests that were performed in the manuscript (Biochemical testing, Serotyping, Antibiotic Sensitivity Testing) I was expecting to see a more diverse array of figures, showcasing the results of those experiments. Instead it appears to be just the PCR results and a flow chart of the study. I know these results are represented more in tables than in figures, but I think it would be useful if the authors added more figures representative of those experiments, to aide the reader in the interpretation of the data.	We have added Figure 4

25	There are a few grammatical errors and phrases that I dont fully understand that are used throughout - for example, "biochemical reactions were PUT UP". I assume that means that "biochemical reactions were CONDUCTED" or a similar phrase. I think this manuscript would benefit from a thorough re-read to remove grammar and spelling issues, as I spotted a not-insignificant amount.	All the grammatic errors were corrected and “PUT UP” was replaced by “CONDUCTED”

VERSION 1

Editor recommendation and comments

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Georgios Efthimiou; University of Hull, Biomedical Science, Hardy Building, Cottingham Road, UNITED KINGDOM, Hull

Date report received: 01 September 2022
Recommendation: Minor Amendment

Comments: The work presented is clear and the arguments well formed. This study would be a valuable contribution to the existing literature. This is a study that would be of interest to the field and community. The reviewers have highlighted minor concerns with the work presented. Please ensure that you address their comments.

Reviewer 2 recommendation and comments

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Jake Barber; Universidad Politecnica de Madrid, SPAIN
https://orcid.org/0000-0001-9279-7763

Date report received: 10 August 2022
Recommendation: Minor Amendment

Comments: Overall I think that the manuscript submitted here is of good quality and suitable for publication at this journal, with a few minor modifications. My general comments are below. General Comments 1) Based on the amount of tests that were performed in the manuscript (Biochemical testing, Serotyping, Antibiotic Sensitivity Testing) I was expecting to see a more diverse array of figures, showcasing the results of those experiments. Instead it appears to be just the PCR results and a flow chart of the study. I know these results are represented more in tables than in figures, but I think it would be useful if the authors added more figures representative of those experiments, to aide the reader in the interpretation of the data. 2) There are a few grammatical errors and phrases that I dont fully understand that are used throughout - for example, "biochemical reactions were PUT UP". I assume that means that "biochemical reactions were CONDUCTED" or a similar phrase. I think this manuscript would benefit from a thorough re-read to remove grammar and spelling issues, as I spotted a not-insignificant amount.

Please rate the manuscript for methodological rigour
Very good

Please rate the quality of the presentation and structure of the manuscript
Good

To what extent are the conclusions supported by the data?
Strongly support

Do you have any concerns of possible image manipulation, plagiarism or any other unethical practices?
No

Is there a potential financial or other conflict of interest between yourself and the author(s)?
No

If this manuscript involves human and/or animal work, have the subjects been treated in an ethical manner and the authors complied with the appropriate guidelines?
Yes

Reviewer 1 recommendation and comments

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Anonymous.

Date report received: 26 July 2022
Recommendation: Minor Amendment

Comments: While up to 1-% of E. coli can be non fermenting, the clinical significance remains largely unknown as they are not routinely screened for role in Diarrheagenic disease. This study has been developed to elucidate the role non fermenting E. coli plays in disease in adult and children populations from 3110 stool samples analysed. 1. Methodological rigour The authors present a number of culture, MALDI-TOF-MS, antimicrobial resistance testing and PCR methods to determine isolates present in the stool samples and for appropriate characterization of microorganisms and resistances present. Appropriate consideration has been employed to differentiate Shigella from non fermenting E. coli. Antimicrobial resistance testing has been performed in line with CLSI guidelines. Molecular assays are well considered for inclusion. 2. Presentation of results Overall the results sections are clear with the percentage of isolates identified in EAEC, EIEC, EPEC and ETEC. Table 2 and 3 are clear. Figures 2 and 3 need modification. An appropriate legend to describe each multiplex would help the reader understand more clearly what is being presented. Also samples on gel images should not be labelled as "negative samples". They should be given a name and stated not detected in the legend. Negative sample may just indicate the assay was not sensitive enough to detect the target of interest. 3. How the style and organization of the paper communicates and represents key findings Style and organization of manuscript are easy to follow and logical. The key findings are clear and address the aims of the study- to elucidate the role that non fermenting E. coli play in diarrheagenic disease. 4. Literature analysis or discussion This study is aimed at a topic that lacks literature. Where appropriate literature exists, it has been referenced. It compares results to a similar study in children in Bangladesh and highlights the differences in study population which may account for some discrepancies in findings which are plausible. 5. Any other relevant comments There are a number of edits which should be considered by the authors: L133- Reference how these methods are performed Section 149- PCR conditions for DEC genes There appears to be an initial PCR mix used followed by a final PCR reaction which contains difference concentrations of MGcl2 and primers. Please clarify which should be used in each instance L152 - "1.5 mM of MgCl2, at a final concentration of 1.5mM"- this is confusing, it is sufficient to state a final concentration. L154- 10μMconcentration should be 10 μM concentration L154- were as follows. Should be were as follows; L157- Amplified samples were tested for 1.5% agarose gel electrophoresis in Tris-borate-EDTA and EtBr staining. Perhaps consider PCR products were subsequently analysed on a 1.5% agarose gel electrophoresis in Tris-borate-EDTA and EtBr staining L160- "were future subjected to" please consider were subsequently tested for L161- remove reference 14 ad it is provided in Table 1 Section 162 PCR for ipaH gene- same comment as for section 149: There appears to be an initial PCR mix used followed by a final PCR reaction which contains difference concentrations of MGcl2 and primers. Please clarify which should be used in each instance L169- Would suggest formatting PCR conditions in same format as lines 155-156 L182-183- states "All EAEC and EIEC isolates were sent to National Salmonella and Escherichia Centre, Central Research Institute, Kasauli (HP), India for Escherichia coli O serotyping". Please reference the method used for serotyping Line 206 - "most common serotype was O22, O88, O149 (4 in number each)" suggest reword to most common serotype was O22, O88, O149 (n=4) Line 207- (3) suggest change to (n=3) Line 217- Please provide a reference to Fischers test L225- "Though NLF E.coli is commonly isolated, they are usually not processed further" - please provide supporting references for this statement. Also place a space between E. and coli L229-231 please provide references for % figures used L237- In the present study,376 please insert space between comma and 376 L255- et al change to et al. L270 "but none of them was immunocompromised" change to but none of them were immunocompromised Throughout text of manuscript Tables and tables are used. Please change table to Table

throughout e.g L200, 218 etc. Table 1: State which is forward, and which is reverse primer Table 1: Please state if primers are in 5' to 3' orientation

Please rate the manuscript for methodological rigour
Very good

Please rate the quality of the presentation and structure of the manuscript
Good

To what extent are the conclusions supported by the data?
Strongly support

Do you have any concerns of possible image manipulation, plagiarism or any other unethical practices?
No

Is there a potential financial or other conflict of interest between yourself and the author(s)?
No

If this manuscript involves human and/or animal work, have the subjects been treated in an ethical manner and the authors complied with the appropriate guidelines?
Yes

SciScore report

https://doi.org/10.1099/acmi.0.000459.v1.1
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iThenticate report

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