Research Article

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Selection and characterization of siderophores of pathogenic *Escherichia coli* intestinal and extraintestinal isolates

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Abstract: Microorganisms adopt a unique mechanism for sequestration and transport of bound iron by producing iron-chelating molecules called siderophores. Siderophores play an important role in colonization of microbes in iron-deficient sites of the host, hence acting as an important virulence factor and promising carriers of antimicrobials to target sites inside the microbial cells. The research was conducted to identify and characterize the siderophores of pathogenic *Escherichia coli* isolates obtained from different parts of India. A total of 534 confirmed *E. coli* isolates were tested for the expression of siderophores using chrome azurol S agar method and its type were determined by Csaky and Arnow assays for hydroxamate and catecholate-type siderophores, respectively. The obtained results revealed a difference in siderophore-type expression by intestinal and extraintestinal pathogenic *E. coli*. On chrome azurol S agar 45.1% of pathogenic *E. coli*. were found to produce siderophores, 12.5% isolates were found to produce catechol-type siderophore and 35.4% isolates produced hydroxamate-type siderophore; and isolates could be classified into five siderotypes (1) only hydroxamate producing (2) only catechol producing (3) both catechol and hydroxamate producing (4) producing siderophores other than hydroxamate and catechol types and (5) siderophore non-producers. Siderophore production was detected in isolates from all geographical regions and in all seasons of the year. The findings conclude that siderophore production is one of the important virulence and epidemiological markers of intestinal as well as extraintestinal pathogenic *E. coli*.

Keywords: siderotyping, ExPEC, EHEC/STEC, hydroxamate siderophore, catecholate siderophore

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1 Introduction

Iron is an essential element for proper metabolism and growth of all living organisms, and bacterial cells require iron in the range of 10^{-7}–10^{-5} M for proper functioning and division; however, free iron availability is limited in host systems; unbound iron concentration in human serum is estimated to be 10^{-24} M; this resulting bacteria experience iron starvation [1,2]. Free iron availability in host is further reduced during infection through nutritional immunity [3]. A tug of war between infecting microorganisms and human host occurs for the limited available bound iron in human tissues and organ systems [4]. Bacteria acquire iron from host iron-bound molecules through various mechanisms; the most important is the siderophore production [5,6]. In pathogenic *Escherichia coli*, siderophore production is considered as one of the major virulent factors helping in the survival and colonization under such stress environment [7–11]. Thus, siderophore production is one of the primary virulence characteristics of pathogenic *E. coli*, which help in its establishment leading to various clinical manifestations ranging from self-limiting diarrhea to fetal childhood diarrhea, urinary tract infection (UTI), hemolytic uremic syndrome (HUS), hemorrhagic colitis (HC), neonatal meningitis, bacteremia, etc. [12,13]. Siderophores are important for iron acquisition and have gained wide applicability in agriculture, environment and medicine [14,15]. In medicine, siderophores are considered useful in drug delivery by Trojan Horse technique, as an anti-infectious agent, an epidemiological tool and a vaccine

candidate [16–18]. Microorganisms are diverse in expressing siderophore types, and various structural types of siderophores have been identified [19]. Two main structural types of siderophore are produced by *E. coli* strains, i.e., catecholate and hydroxamate types [5]. In the present study, intestinal and extraintestinal clinical *E. coli* isolates from various parts of India were evaluated to detect siderophore production, the prevalence of siderophore-producing *E. coli* and the diversity of pathogenic *E. coli* based on the type of siderophores.

2 Materials and methods

2.1 Study samples

A total of 783 suspected intestinal and extraintestinal *E. coli* isolates obtained from the National Salmonella and Escherichia Centre (NSEC), Central Research Institute Kasauli (Himachal Pradesh) from January 2016 to January 2018 were initially considered for the study. In all, 534 isolates (302 intestinal – human diarrhea and 232 extraintestinal – human UTI) confirmed as *E. coli* on biochemical characterization were considered for further study. These isolates were obtained from various geographical regions of the country (59 Central India, 134 Eastern India, 148 Northern India, 170 Southern India and 23 Western India). On the basis of seasonality, 534 total study isolates could be grouped as 97 summer isolates, 310 monsoon isolates, 25 post-monsoon isolates and 102 winter isolates.

2.2 Biotyping

Isolates were confirmed as *E. coli* on the basis of biotyping performed as described by Edwards and Ewing [20]. Briefly, isolates were examined for bacterial growth characteristics on MacConkey agar, nutrient agar and nutrient broth. They were microscopically examined (Quasmo Microscopes & Micro Lenses) after Gram's staining to study the bacterial morphology and subjected to a set of biochemical tests including sugar fermentation (lactose, glucose, sucrose), indole, methyl red, Voges–Proskauer, citrate utilization (IMViC), urea utilization, triple sugar iron, nitrate reduction, oxidase test, catalase test, and *ortho*-nitrophenyl beta-D-galactopyranoside. Isolates giving characteristic reaction consistent with *E. coli* were considered as confirmed *E. coli*. Bacteriological media and reagents were prepared using dehydrated media and chemicals of reputed manufacturers, i.e., Hi-media Biosciences Company, Microexpress, BDH, E-Merck and SRL P. Ltd.

2.3 Serotyping

All confirmed *E. coli* isolates were typed using standard anti-"O" *E. coli* antiserum as described by Orskov and Orskov [21]. Briefly, the organisms to be typed were grown in nutrient broth (Micromaster Lab Pvt. Ltd.) overnight at 35 ± 2°C. The bacterial growth was boiled for 1 h and formalin was added to the final concentration of 0.25% (Gen Chem Fine Chemicals). Suspension was diluted to a concentration of 3×10^9 organisms/mL using normal saline as checked using Brown's opacity set. This was used as an antigenic suspension, which was allowed to first react with pooled anti-*E. coli* O antisera (provided by NSEC, Central Research Institute, Kasauli, India) in a U-shaped microtiter plate at 35 ± 2°C overnight and agglutination was observed. Monovalent antisera of the pool that gave clear agglutination were then tested again with the test suspension for agglutination to finalize the "O" serogroup. Negative control was included in each set of the reaction by adding normal saline and test suspension in the negative control wells. Isolates not agglutinating with any of the antiserum were designated as UT (un-typeable).

2.4 Siderophore screening

Production of siderophores by *E. coli* isolates was screened on chrome azurol S (CAS) agar [22,23] by inoculating CAS (Hi-media Biosciences Company) agar with the test strain. The CAS agar plates were prepared as described earlier [24]. Inoculated CAS agar plates were incubated at 35 ± 2°C for 48 h and were scored siderophore positive when the color change from blue to orange was observed around the inoculated area.

2.5 Siderophore characterization

All *E. coli* isolates were chemically characterized to detect the two most commonly produced siderophore types by using chemical assays described by Csaky for hydroxamate-type siderophore detection and Arnow for detection

of catecholate-type siderophore [25–27]. To express siderophores, the test strains were grown under iron starvation conditions in iron-restricted modified Fiss Minimal Medium. Culture supernatant of the test isolates grown overnight in minimal medium was tested using Csaky and Arnow assays for hydroxamate-type and catecholate-type siderophores, respectively. For hydroxamate detection, 1 mL of culture supernatant was hydrolyzed with 1 mL of 6 M H_2SO_4 and autoclaved for 30 min. To this 3 mL of sodium acetate (35%), 1 mL of sulfanilic acid (1% in 30% acetic acid) and 0.5 mL of iodine solution (1.3% in 30% acetic acid) were added. After 5 min, the excess iodine was destroyed with 1 mL of sodium arsenite solution (2% w/v). One milliliter of naphthylamine (0.3% in 30% acetic acid) was then added and allowed for 20 min to develop pink color. Absorbance was measured at 526 nm (Hitachi U-3010 UV-Visible spectrophotometer) using deionized water as blank.

For catechol detection, to 1 mL of culture supernatant 1 mL of HCl was added followed by 1 mL of nitrite–molybdate and then 1 mL of 1 N NaOH was added to develop the color (color changes to red in positive cultures). Absorbance was measured at 510 nm (Hitachi U-3010 UV-Visible spectrophotometer) using deionized water as blank.

All the reagents were prepared using chemicals of reputed manufacturers, i.e., SD Fine chemicals, Hi-media Biosciences Company, BDH and E-Merck.

2.6 Statistical analysis

Mean and standard deviations of five absorbance readings of each reaction in chemical assays for siderophore detection and all data processing for frequencies, percentages, associations and significances using chi-square test (95% confidence level) were calculated using SPSS software version 22.0.

3 Results

3.1 Siderophore screening on CAS agar

All biochemically confirmed 534 *E. coli* isolates (302 intestinal and 232 extraintestinal) were screened for the production of siderophores on CAS agar. Siderophores were detected in isolates from all parts of the country.

Figure 1: Percentage of siderophore producers (%) among pathogenic intestinal and extraintestinal *E. coli* isolates.

Siderophores were detected in 45.1% of the isolates as detected by culture on CAS agar. Percentage of siderophore production was greater in extraintestinal *E. coli* isolates than in the intestinal isolates ($p < 0.05$) (Figure 1). Among the total *E. coli* isolates, the percentage of siderophore producers was found to be greater among the isolates from Southern, Eastern and Central India than from Western and Northern India ($p < 0.05$) (Table 1).

3.2 Siderotyping by chemical characterization

Of the 241 siderophore-positive screened isolates on CAS agar, 53 (22%) were found to be negative in chemical characterization for hydroxamate- and catechol-type siderophores. On the other hand, catechol- or hydroxamate-type siderophores were detected in 62 (21.2%) of 293 CAS-negative isolates (17.4% hydroxamate, 3.8% catechol) (Table 2). Catechol- or hydroxamate-type siderophores in some CAS agar-negative isolates were detected in isolates belonging to different "O" serogroups (Table 2). Thus, after chemical characterization, a total of 56.7%

Table 1: Region-wise distribution of siderophore-producing *E. coli* isolates

Geographical area	Siderophore detection by CAS agar method		Total no. of isolates
	Siderophore producers	Siderophore nonproducers	
Central India	31 (52.5%)	28 (47.5%)	59
Eastern India	75 (56%)	59 (44%)	134
Northern India	33 (22.3%)	115 (77.7%)	148
Southern India	99 (58.2%)	71 (41.8%)	170
Western India	3 (13%)	20 (87%)	23
Total	241 (45.1%)	293 (54.9%)	534

Table 2: Comparative evaluation of siderophore production in intestinal and extraintestinal *E. coli*

Source	Among CAS agar-positive isolates				Among CAS agar-negative isolates			Overall		
	n = 241 (intestinal: 110, extraintestinal: 131)				*n* = 293 (intestinal: 192, extraintestinal: 101)			*n* = 534		
	1	2	3	4	5	6	7	8 (1 + 3 + 5)	9 (2 + 3 + 6)	10 (8 + 9 + 4 − 3)
	Only hydroxamate detected	Only catechol detected	Both hydroxamate and catechol detected	None of hydroxamate and catechol detected	Only hydroxamate detected	Only catechol detected	Both hydroxamate and catechol detected	Hydroxamate detected	Catechol detected	Siderophores detected
Intestinal	76 UT (16), O1 (1), O2 (2), O5 (1), O7 (1), O8 (6), O9 (2), O11 (4), O16 (1), O17 (3), O20 (1), O22 (11), O34 (2), O35 (1), O83 (2), O88 (3), O98 (1), O119 (1), O126 (9), O128 (3), O141 (2), O149 (2), O157 (1)	5 UT (1), O22 (1), O88 (2), O157 (1)	1 O7 (1)	28 UT (2), O2 (3), O7 (2), O8 (1), O11 (1), O20 (1), O22 (5), O35 (1), O83 (1), O101 (1), O119 (1), O126 (4), O141 (5)	32 UT (10), O2 (2), O8 (7), O20 (2), O22 (1), O88 (2), O98 (1), O119 (2), O126 (1), O128 (3), O141 (1)	4 UT (1), O88 (1), O149 (1), O157 (1)	0	**109**	**10**	**146**
Extraintestinal	56 UT (13), O1 (2), O2 (2), O7 (1), O8 (5), O9 (1), O11 (6), O17 (1), O20 (5), O22 (1), O35 (2), O63 (1), O83 (1), O88 (6), O117 (1), O118 (1), O141 (5), O149 (2)	45 UT (2), O7 (6), O8 (7), O11 (8), O16 (1), O17 (2), O34 (2), O35 (3), O83 (3), O88 (3), O89 (1), O101 (1), O119 (1), O141 (4), O149 (1)	5 O7 (1), O11 (1), O35 (1), O83 (1), O88 (1)	25 UT (4), O7 (6), O8 (1), O11 (6), O20 (1), O35 (6), O141 (1)	19 UT (1), O1 (1), O2 (1), O4 (1), O7 (1), O8 (2), O11 (1), O22 (2), O34 (1), O35 (1), O88 (1), O101 (1), O141 (3), O149 (1), O157 (1)	7 UT (1), O4 (1), O8 (1), O20 (1), O49 (1), O83 (2)	0	**80**	**57**	**157**
Total	**132**	**50**	**6**	**53**	**51**	**11**	**0**	**189**	**67**	**303**

(303 of 534) isolates were siderophore producers while 43.3% (231 of 534) were found to be siderophore nonproducers.

On chemical characterization, *E. coli* isolates could be categorized as only hydroxamate-type siderophore producers 34.4% (183 of 534), only catechol-type siderophore producers 11.4% (61 of 534), both hydroxamate- and catecholate-type siderophore producers 1.1% (6 of 534), other than hydroxamate- and catecholate-type siderophore producers 9.9% (53 of 534) and siderophore nonproducers 43.3% (231 of 534). Hydroxamate-type siderophores were found to be more frequently expressed by *E. coli* isolates ($p < 0.005$); by taking into account both catechol and hydroxamate siderophore producers, a total of 12.5% (67 of 534) isolates were found to produce catechol-type siderophores and 35.4% of the isolates was found to produce hydroxamate-type siderophores. Catechol-type siderophore-producing isolates were of 12.5% (11.4% produced catechol type alone while 1.1% produced both catechol and hydroxamate siderophores) and hydroxamate-type siderophore-producing isolates were of 35.4% (189 of 534; i.e., 34.4% produced hydroxamate-type siderophore alone while 1.1% produced both catechol and hydroxamate siderophores; Table 2). Within intestinal isolates, the frequency of detection of hydroxamate-type siderophore was more than the catechol-type siderophore ($p < 0.005$); 36.1% (109/302) of the intestinal isolates produced hydroxamate-type siderophore while only 3.3% (10/302) produced catechol-type siderophore. Within extraintestinal, both types of siderophores were detected in very large percentage of isolates (34.5%, 80 of 232 hydroxamate type and 24.6%, 57 of 232 catechol type). Although the hydroxamate-type siderophore was detected in both intestinal and extraintestinal *E. coli* isolates with no significant difference in the frequency of detection ($p > 0.05$); catechol-type siderophore was detected more frequently among extraintestinal isolates than in intestinal isolates ($p < 0.05$). Six isolates (one intestinal and five extraintestinal) were found to produce both types of siderophores. Some of the CAS agar-negative isolates were also found to produce siderophores in chemical assays (Table 2 and Figure 2), which may probably be attributed to assay limitations of CAS agar method as pointed out by Shin et al. [23]. Hydroxamate-type siderophore-producing *E. coli* isolates were found to be the most frequent in all geographical areas ($p < 0.05$) (Figure 3).

Expression of siderophores was not found uniform among various *E. coli* "O" serogroups ($p < 0.005$). Isolates in all the "O" serogroups were not found to produce siderophores as all of the isolates with serogroups O84 (7/7), O86 (2/2), O114 (1/1), O116 (2/2), O120 (4/4) and autoagglutinating (4/4) did not give positive results on CAS agar, and catechol and hydroxamate were also not detected in any of these serogroups while all isolates in some serogroups were found to produce siderophores, i.e., O5 (1/1), O16 (2/2) and O117 (1/1). The frequency of siderophore production was quite high in some of the serogroups, i.e., O7 (85.7%), O11 (68.4%), O34 (80%), O35 (56%) and O141 (73.9%). However, it was only found significant in O7, O11 and O141 ($p < 0.05$). No significant association was observed between "O" serogroup and type of siderophore. Very few isolates (6/534; 1.1%) were found to produce both catechol and hydroxamate type of siderophores, i.e., O7 (2/21), O11 (1/38), O35 (1/25), O83 (1/25) and O88 (1/33) (Table 3).

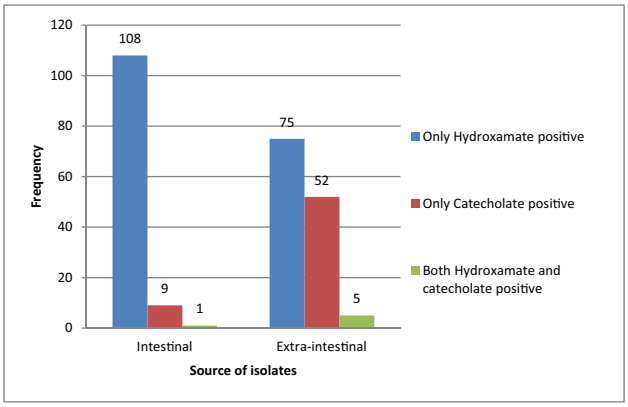

Figure 2: Frequency of siderophore types among pathogenic *E. coli* isolates.

It was observed that *E. coli* isolates from summer (84.5%; 82/97) and monsoon/post-monsoon (51.6%; 160/310 and 88%; 22/25) seasons produced siderophores more frequently than from winter season (38.2%; 39/102) ($p < 0.05$). Same pattern was observed in case of intestinal as well as extraintestinal isolates (Table 4).

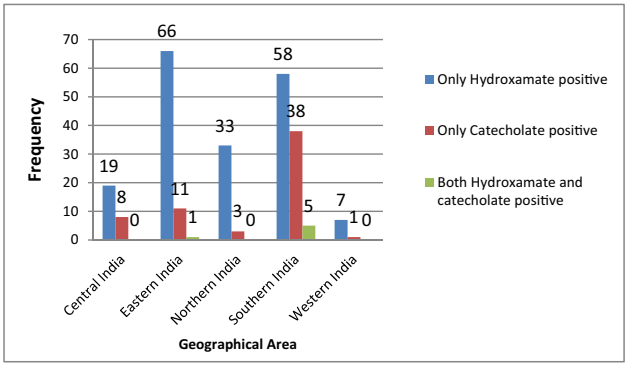

Figure 3: Geographical frequency of siderophore types.

Table 3: Expression of siderophores and siderophore types by various *E. coli* "O" serogroups

"O" Serogroup	Siderophore screening on CAS agar *N* (%)			Hydroxamate/catechol detection *N* (%)				
	Positive	Negative	Total	Hydroxamate positive	Catechol positive	Both positive	Both negative	Total
UT	38 (39.6)	58 (60.4)	96	40 (41.7)	5 (5.2)	0	51 (53.1)	96
O1	3 (60)	2 (40)	5	4 (80)	0	0	1 (20)	5
O2	7 (43.8)	9 (56.3)	16	7 (43.8)	0	0	9 (56.3)	16
O4	0	3 (100)	3	1 (33.3)	1 (33.3)	0	1 (33.3)	3
O5	1 (100)	0	1	1 (100)	0	0	0	1
O7	18 (85.7)	3 (14.3)	21	3 (14.3)	6 (28.6)	2 (9.5)	10 (47.6)	21
O8	20 (43.5)	26 (56.5)	46	20 (43.5)	8 (17.4)	0	18 (39.1)	46
O9	3 (50)	3 (50)	6	3 (50)	0	0	3 (50)	6
O11	26 (68.4)	12 (31.6)	38	11 (29)	8 (21)	1 (2.6)	18 (47.4)	38
O16	2 (100)	0	2	1 (50)	1 (50)	0	0	2
O17	6 (46.2)	7 (53.8)	13	4 (30.8)	2 (15.4)	0	7 (53.8)	13
O20	8 (40)	12 (60)	20	8 (40)	1 (5)	0	11 (55)	20
O22	18 (54.5)	15 (45.5)	33	15 (45.5)	1 (3)	0	17 (51.5)	33
O34	4 (80)	1 (20)	5	3 (60)	2 (40)	0	0	5
O35	14 (56)	11 (44)	25	4 (16)	3 (12)	1 (4)	17 (68)	25
O49	0	5 (100)	5	0	1 (20)	0	4 (80)	5
O63	1 (33.3)	2 (66.7)	3	1 (33.3)	0	0	2 (66.7)	3
O83	8 (32)	17 (68)	25	3 (12)	5 (20)	1 (4)	16 (64)	25
O84	0	7 (100)	7	0	0	0	7 (100)	7
O86	0	2 (100)	2	0	0	0	2 (100)	2
O88	15 (45.5)	18 (54.5)	33	12 (36.4)	6 (18.2)	1 (3)	14 (42.4)	33
O89	1 (14.3)	6 (85.7)	7	0	1 (14.3)	0	6 (85.7)	7
O98	1 (50)	1 (50)	2	2 (100)	0	0	0	2
O101	2 (25)	6 (75)	8	1 (12.5)	1 (12.5)	0	6 (75)	8
O114	0	1 (100)	1	0	0	0	1 (100)	1
O116	0	2 (100)	2	0	0	0	2 (100)	2
O117	1 (100)	0	1	1 (100)	0	0	0	1
O118	1 (50)	1 (50)	2	1 (50)	0	0	1 (50)	2
O119	3 (30)	7 (70)	10	3 (30)	1 (10)	0	6 (60)	10
O120	0	4 (100)	4	0	0	0	4 (100)	4
O126	13 (46.4)	15 (53.6)	28	10 (35.7)	0	0	18 (64.3)	28
O128	3 (37.5)	5 (62.5)	8	6 (75)	0	0	2 (25)	8
O141	17 (73.9)	6 (26.1)	23	11 (47.8)	4 (17.4)	0	8 (34.8)	23
O149	5 (22.7)	17 (77.3)	22	5 (22.7)	2 (9.1)	0	15 (68.2)	22
O157	2 (28.6)	5 (71.4)	7	2 (28.6)	2 (28.6)	0	3 (42.9)	7
Autoagglutinating	0	4 (100)	4	0	0	0	4 (100)	4

4 Discussion

Siderophores are one of the important virulence factors that help survival of *E. coli* in iron-starvation conditions in the host by sequestration and transmission of iron for its proper multiplication [7–11]. In the present study, a very large number of *E. coli* isolates (41.5%) were found to be siderophore producers of a total 534 confirmed *E. coli* isolates based on screening using CAS agar technique, thus indicating siderophore production as one of the important virulence factor in pathogenic *E. coli*. The percentage of siderophore producers was found to be greater among isolates from Southern, Eastern and Central India than from Western and Northern India. However, in similar studies carried out by other researchers in Western and Northern India, siderophore production was detected in a very large percentage of isolates from these regions of the country [28,29]. Thus, siderophore production seems to be an important virulence characteristic among *E. coli* isolates prevalent in all geographical areas in India. Siderophore production on CAS agar screening among extraintestinal isolates (131 of 232, 56.5%) was found to be more ($p < 0.05$) than those among intestinal isolates (110 of 302, 36.4%). Siderophore production has

Table 4: Comparative evaluation of siderophores produced by *E. coli* isolates in different seasons

Seasons	Among CAS agar-positive isolates				Among CAS agar-negative isolates			Overall		
	n = 241 (summer: 72, winter: 33, monsoon: 122, post-monsoon: 14)				*n* = 293 (summer: 25, winter: 69, monsoon: 188, post-monsoon: 11)			*n* = 534 (summer: 97, winter: 102, monsoon: 310, post-monsoon: 25)		
	1	2	3	4	5	6	7	8 (1 + 3 + 5)	9 (2 + 3 + 6)	10 (8 + 9 + 4 − 3)
	Only hydroxamate detected	Only catechol detected	Both hydroxamate and catechol detected	Neither hydroxamate nor catechol detected	Only hydroxamate detected	Only catechol detected	Both hydroxamate and catechol detected	Hydroxamate detected	Catechol detected	Siderophores detected
Summer	28 Intestinal: 17 ExPEC: 11	22 Intestinal: 1 ExPEC: 21	6 Intestinal: 1 ExPEC: 5	16 Intestinal: 8 ExPEC: 8	5 Intestinal: 4 ExPEC: 1	5 Intestinal: 1 ExPEC: 4	0	**39**	**33**	**82** Intestinal: 32 ExPEC: 50
Winter	24 Intestinal: 22 ExPEC: 2	3 Intestinal: 2 ExPEC: 1	0	6 Intestinal: 6 ExPEC: 0	6 Intestinal: 1 ExPEC: 5	0	0	**30**	**3**	**39** Intestinal: 31 ExPEC: 8
Monsoon	70 Intestinal: 27 ExPEC: 43	24 Intestinal: 1 ExPEC: 23	0	28 Intestinal: 11 ExPEC: 17	32 Intestinal: 19 ExPEC: 13	6 Intestinal: 3 ExPEC: 3	0	**102**	**30**	**160** Intestinal: 61 ExPEC: 99
Post-monsoon	10 Intestinal: 10 ExPEC: 0	1 Intestinal: 1 ExPEC: 0	0	3 Intestinal: 3 ExPEC: 0	8 Intestinal: 8 ExPEC: 0	0	0	**18**	**1**	**22** Intestinal: 22 ExPEC: 0
Total	**132**	**50**	**6**	**53**	**51**	**11**	**0**	**189**	**67**	**303**

ExPEC: extraintestinal pathogenic *Escherichia coli*.

been found to be strongly associated with extraintestinal *E. coli* in various other studies also [11,30].

Two main structural types of siderophores are produced by *E. coli* strains, i.e., catecholate and hydroxamate types [5]. In the present study, both of these siderophore types were detected in 47.9% (256 of 534) isolates including 11.6% (62/534) isolates which were found to be negative in screening with CAS agar culture technique. Hydroxamate-type siderophore was found to be more frequently expressed (35.4%) than catechol type (12.5%) among the total 534 *E. coli* isolates studied ($p < 0.05$). Hydroxamate-type siderophore was found to be more frequently expressed by both intestinal (36.1%) and extraintestinal (34.5%) isolates ($p < 0.05$). However, a difference was observed in the frequency of catecholate-type siderophore production among intestinal and extraintestinal isolates ($p < 0.05$) as 24.6% of extraintestinal isolates was catecholate positive while only 3.3% of the intestinal *E. coli* isolates was found to be producing catecholate-type siderophore. In earlier studies, both these siderophore types (hydroxamate and catecholate) have been shown to be the major types in intestinal and extraintestinal *E. coli*, and catechol-type siderophore has been recognized as an important virulent factor associated with extraintestinal *E. coli* [7,31,32]. The results of the present study are, thus, consistent with the earlier studies.

In 9.9% (53 of 534) isolates which were found to be producing siderophore in CAS agar screening, hydroxamate and catechol could not be detected. Thus, indicating the expression of siderophores other than the two commonly expressed types by these isolates. Taking these into account, the overall siderophore producers in the present study were 56.7% (303 of 534). Bacteria including *E. coli* have been found to be diverse in terms of expression of siderophores [33,34]. This diversity of siderophore production in bacterial isolates can be applied as an important epidemiological tool for typing of these isolates [5,35]. In the present study, 534 *E. coli* isolates were grouped into 36 "O" serogroups which could further be sidero-typed into five categories, viz., (1) only hydroxamate producing, (2) only catechol producing, (3) both catechol and hydroxamate producing, (4) producing siderophores other than hydroxamate and catechol types and (5) siderophore nonproducers. In the present study, isolates in which no siderophore was detected may be using other mechanisms for iron acquisition such as direct utilization of iron-bound host molecules, enzymes for the release of iron from iron complex molecules or hemolytic cytotoxins to free iron from hemoglobin or heme [36,37].

Isolates in the present study belonged to 36 different serological groups including un-typeable and autoagglutinating, with nonuniform frequency of "O" serogroups (some being represented by more isolates while some being represented only by a single isolate). Significant association between "O" serogroup and siderophore production was only found in few serogroups, viz., O7, O11 and O141 ($p < 0.05$), more frequently in extraintestinal *E. coli* isolates. These serogroups are frequently associated with extraintestinal *E. coli* infections [38]. Based on the expression of siderophores by a very large number of serogroups in the present study, serogroups and siderophores seem to be independently expressed virulence traits.

The importance of siderophores as one of the virulence factors is also recognized in pathogenic enterohemorrhagic *E. coli* (EHEC/STEC) strains [39,40]. *E. coli* serogroup O157 is frequently associated with the most virulent-type EHEC/STEC infections, i.e., HC, HUS [41,42]. In the present study, 57% (4/7) of *E. coli* O157 isolates was also found to produce siderophores (two hydroxamate types and two catechol types). This suggests the importance of siderophores in EHEC/STEC infections.

The percentage of siderophore expression was greater in isolates from summer (84.5%; 82/97) and monsoon/post-monsoon (51.6%; 160/310 and 88%; 22/25) seasons than from winter season (38.2%; 39/102) ($p < 0.05$). Bacterial infections including those caused by *E. coli* are seasonal, i.e., being more predominant during summer, monsoon and post-monsoon seasons especially in poor socioeconomic countries [43,44] as they can better transmit during these seasons. Expression of virulence factors, including siderophore production, further contributes to their better colonization and establishment when infecting the animal or human host.

Detection of siderophores in 56.7% (303/534) clinical *E. coli* isolates in the present study, diversity in the expression of siderophore types by these isolates, detection of siderophores in clinically important serogroups including *E. coli* O157 and seasonal trends in siderophore expression clearly indicate the importance of siderophore expression in *E. coli* virulence and epidemiology.

5 Conclusion

It can be concluded from the present study that siderophore production is an important virulence characteristic of pathogenic *E. coli* as we detected 41.1% siderophore-producing isolates in screening and overall 56.7% during

characterization. Detection of siderophores in *E. coli* isolates from all geographical regions and its detection in large percentage during summer (84.5%) and monsoon/post-monsoon (51.6 and 88%) seasons indicate the importance of siderophores in *E. coli* epidemiology. Siderotyping can also be an useful important typing tool for the characterization of *E. coli* isolates, and we could type *E. coli* isolates into five different sidero-types, viz., (1) only hydroxamate producing, (2) only catechol producing, (3) both catechol and hydroxamate producing, (4) producing siderophores other than hydroxamate and catechol types and (5) siderophore nonproducers. The results of present study show siderophore expression as an important *E. coli* virulence and epidemiology marker.

Abbreviations

CAS	chrome azurol S
EHEC	enterohemorrhagic *Escherichia coli*
ExPEC	extraintestinal pathogenic *Escherichia coli*
HC	hemorrhagic colitis
HUS	hemolytic uremic syndrome
NSEC	National Salmonella and Escherichia Centre
STEC	Shiga toxin-producing *Escherichia coli*

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