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# Molecular characterization of colicinogenic *E.coli* inhibitory to *E.coli* O157:H7 and *E.coli* O26:H11

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**Key words:** *Escherichia coli*, Colicin, Bacteriocin, Virulence factor, Probiotics.

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## Abstract

Bacteriocinogenic microbial species are considered as an emerging alternative to antibiotics due to their enhanced prophylaxis against invading pathogens. Commensal *E.coli*, from human, cattle and sheep, was examined for its ability to competitively exclude closely related species *in-vitro* and the antimicrobials released were analyzed. *E.coli* isolates (n=513) screened for anti–*E.coli* O157:H7 and O26:H11 activity *in-vitro* and the release of colicins. Among all, 9.3% colicinogenic *E.coli* inhibited the growth of these pathogen species. Colicin gene detection showed that col E6 (77%) andIb (64%) were most frequently occurring colicins in inhibitory *E.coli* followed by colE7 (52%), E4 (58%),Ia (27%), J (37%), M (27%), S4(18%) and E3 (18%). Other colicins (col A, D, E1, E2, E5, E8, E9 and col 10) were less frequently detected whereas col B, K and 5 were not detected in any *E.coli* isolates. Phylogeney of these colicinogenic *E.coli* classified isolates as 47% B2, 16% B1, 16% D1, 10% A1 and 8.3% Ao *E.coli*. Virulence gene detection and 16SrDNA sequencing confirmed15 non-pathogenic *E.coli* strains which also showed sensitivity to commonly used antibiotics and were lacking siderophore activity.The study concludes that the potential role of *E.coli* in human and animalgut is to competitively exclude invading pathogens. Thus, non-virulent colicininogenic*E.coli* can be suggested for further detailed studies, both *in-vitro* and *in-vivo*, to be used as probiotics.

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## Introduction

Pathogenic strains of *E.coli* have been categorized into various types on the basis of the site of infection, pathogenicitymode and presence of virulence genes. Enterohemorrhagic *E. coli* O157:H7 and enterotoxigenic *E. coli* O26:H11 have reportedly caused severe gastero-intestinal disease outbreaks in individuals of United States and Europe (Brashears *et al.*, 2003) while recently prevalence of these pathogens have been raised in Asia as well (Ingle *et al.*, 2018). Epidemiological studies showed that cattle and bovineare the potential carriers of these pathogens. According to certain case studies, consumption of contaminated water, fruits or vegetables with wastes of these animals have resulted into severe infections (Willyard, 2017). Strategies have been designed to minimize fecal shedding of such pathogens from animals and trial experiments are still under practice. Feeding cattle with a specific dose of beneficial bacteria, called probiotics is a new promising approach. Lactobacteriaceae, non-pathogenic bacilli and *E.coli* strains have been commercialized as probiotic supplements which provide several health benefits (Collado *et al.*, 2009). Probiotic *E.coli* fed to neonatal calves resulted in reduced faecal shedding of enterohemorrhagic *E.coli* (Zhao *et al.*, 1998). Conventionally, the release of antimicrobial peptides called bacteriocins, by the probiotic strains has been considered as an important element of interspecies competition inside the host body (Cascales *et al.*, 2007).In poultry and swine, competitive exclusion by probiotic preparations has eliminated *Salmonella* species and enterotoxigenic *E.coli* but in cattle, only few Lactobacillus species have caused *E.coli* O157:H7inhibition (Nedialkova *et al.*, 2014). The inhibitory property of *E.coli* colicins has also been stated in different studies. The growth of *E.coli* O157:H7 was suppressed by 18 of 1200 colicinogenic *E.coli in-vitro,* which were isolated from cattle rumen (Smarda and Obdrzalek, 2001). Additionally, purified colicins (G, H, E2 and E7) spotted on media plates seeded with single colony of pathogens (*Salmonella enterica* and *E.coli* O157:H7) inhibited the growth which appeared as halo zones (Schulz *et al.*, 2015).

The current study was carried out to identify colicinogenic *E.coli* isolates against *E.coli* O157:H7 and O26:H11 from different hosts i.e. human, cow and sheep. Further, they were characterized for type of antimicrobial peptide produced, phylogenetic groups, virulence, antibiotic sensitivity and hemolytic property. It would help to identify potentially useful strains that can control pathogenic *E.coli* in livestock and humans.

## Material and methods

*Isolation of E.coli isolates*

Commensal *E.coli* isolates were isolated from three hundred faecal specimens of uninfected human, cow and sheep. Each sample was initially homogenised in normal saline (1:10 dilution), followed by centrifugation at 5000g for 15 minutes to pellet the heavy wastes. The supernatant (100µl) was spread onto sterile MacConkey media plates (Oxoid: CM0007) and incubated for 24hours at 37°C. Since *E.coli* is Lactose Fermenting (LF), pink coloured LF colonies were randomly selected from initial cultures and were sub-cultured to obtain pure colonies. Further Gram staining and biochemical tests (20 E API, BioMérieux, France) were performed to confirm purified colonies as *E.coli* while other species were not included in the study.

*Control and indicator strains used in the study*

A set of indicator species of gram negative pathogens and controls for different procedures are given in Table 1a. Cultures of all strains were grown and maintained on Luria Bertani (Sigma-Aldrich: L3522) media.

*Colicin sensitivity detection method*

Colicin production was determined as described by previously (Smajs *et al.*, 2010). Briefly, each producer strain was grown in four parallel broths at cells density of $10^3$ cells/100ul i.e. i) TS(Tryptic Soy Broth, CM0129 Oxoid) supplemented with Mitomycin C (0.01%), ii)TS supplemented with Proteinase K (0.1%, w/v), iii) TS culture non-supplemented and iv) LB culture. After 48 hours incubation, two ml of each culture were transferred to Eppendroff tubes and

centrifuged for five minutes at 10,000g to harvest cells. The pellet was diluted with two ml sterile TS broth and 50ul/ml of chloroform and then was thoroughly mixed by vortex. Mixture tubes were kept open for 30 minutes at room temperature to lyse cell and release of colicins.

For agar well diffusion assay, LB agar was pre-seeded with an indicator strain ($10^7$ cells/100ml) and five wells (seven millimetre diameter) were bored into solidified media plates. Chloroform treated cell free supernatant (100ul) of each *E.coli* isolates was poured into individual wells (four culture types in four wells) while control culture preparation in the centred well. Duplicate culture plates were performed for each isolate and were incubated for 24hours at 37°C. Zone of inhibition formed due to release of colicins by producer species and growth inhibition of indicator species was measured.

*Molecular detection of colicin gene determinants*

*Colicin producing E.coli* isolates that were able to inhibit the growth of indicator strains retained multiple colicin types. Template DNA was extracted from fresh LB cultures of each isolate by using Get-Genomic DNA purification system (Thermo-Fischer Scientific: K0512). The primers and PCR conditions for all colicins were used as described previously using specific gene primers (Table 1b) (Smajs *et al*., 2010, Toshima *et al*., 2008). PCR reaction solution was prepared by adding 12µl DreamTaqmaster mix, 1.5µl of each primer (Forward and Reverse),5µl of sample DNA in 5µl of PCR grade water. The amplified products were visualized using standard 1% Agarose gel electrophoresis procedure.

*Distribution of E.coli isolates in phylogenetic groups*

Colicinogenic *E.coli* were classified into specific phylogenetic group (A0, A1, B1, B2, D1 and D2) based on presence of three characterizing genes i.e. *yja*A, *chu*A and TspE4.C2 (Table 1b). For the detection of these genes, triplex PCR was carried out using specific primers and PCR conditions given by Clermont *et al*., 2000. The preparation of reaction mixture and visualizing gene product was done as described previously.

*Detection of virulence genes*

The presence of virulence genes in microbial species indicates the pathogenic nature of organism. Thus, uni-plex PCR was carried out for the detection of five virulence genes i.e. Shiga toxins (*stx*1 and *stx*2), intimin (*eae*A), enterohemolysin (*hly-α*) and heat stable toxin (*St*), in colicinogenic *E.coli*. The PCR primers and conditions are given in Table 1b (Tharmaraj and Shah, 2009).

*Identification of E.coli strains*

*E.coli* isolates lacking all of the five virulence genes were selected for further molecular identification on the basis of 16SrRNA sequence. DNA samples of all isolates were sent to Macrogen, South Korea, where the sequence was amplified using universal primers 27F and 1492 R. The 16SrDNA sequence of each *E.coli* isolate obtained was carefully analysed and compared to previously reported sequences of *E.coli* strains.

*Antibiotic sensitivity assay*

The identified *E.coli* strains were evaluated for their response (sensitive or resistant) to commonly used antibiotics. Antibiotic susceptibility test was performed with the standard disk diffusion assay (James and Biemer, 1973) while antibiotics included: Amikacin 30ug, ampicillin 10 mg, cefotaxime 30ug, chloramphenicol 30 mg, ciprofloxacin 5 mg, Nalidixic acid 30ug, meropenem/imipenem 10ug tetracycline30 mg, streptomycin 10ug and sulfamethoxazole- trimethoprim 25 mg. Overnight cultures of test organisms in TS media were used to carry out assay on Mueller- Hinton agar media (Difco). After 24 hours incubation at 37°C, inhibitory zones formed around the discs were measured and compared to antibiotic sensitivity standards given by CLSI (NCLS, 2000).

*Siderophore assay*

Each colicinogenic *E.coli* was checked for its ability to bind iron in its surrounding. A detection method developed by Schwyn and Neilands, 1987, was used in which overnight cultures of colicinogenic *E.coli* in TS broth were pelleted and suspended in fresh broth.

From fresh culture, 10ul was spotted on the surface of chrome azurol S agar plates for overnight incubation. A positive result for siderophore production was indicated by an orange halo surrounding the colony.

**Results**

*Antagonistic activity of colicinogenic E.coli isolates*

Altogether, 513 *E.coli* 1/ 2 were obtained from 300 samples, where *E.coli* 1 and 2 were present in a proportion of 34:25 in cow (n=177), 72:83 in sheep (n=155) and 97:84 in humans (n=181) (Fig.1). Among these isolates, 9.3% *E.coli* were found having ability to release growth inhibitory colicins against *E.coli* O157:H7 and O26:H11.

**Table 1a.** List of indicator species and controls used in the study.

| Property | Control |
|---|---|
| Indicator Specie | *E.coli* O157:H7 |
| Indicator Specie | *E.coli* O26:H11 |
| Colicin A | *E. coli* BZB2101pColA - CA31 |
| Colicin B | BZB2102 pColB - K260 |
| Colicin D | BZB2103 pColD - CA23, |
| Colicin E1 | *E. coli* 385/80 pColE1 |
| Colicin E2 | pColV*E. coli* 189BM pColE2 - P9, |
| Colicin E3 | *E. coli* 185 M4 pColE3 - CA38 |
| Colicin E6 | BZB2150 pColE6 - CT14 |
| Colicin E7 | BZB2120 pColE7 - K317 |
| Colicin K | BZB2116 pColK - K235 |
| Colicin M | PAP1 pColM - BZBNC22 |
| Colicin Ia | BZB2279 pColIa - CA53 |
| Colicin Ib | BZB2202 ColIb – P9 |

The zones of inhibition were measured in millimeters where >70% species showed minimum (5mm) to moderate (7mm) activity during incubation time of 24 hours while <10% showed strong (10mm) inhibition activity. *E.coli* O157:H7 was inhibited by 47.9% of *E.coli* isolates while growth reduction of *E.coli* O26:H11 was done by 43.7% isolates. Approximately, 8.3% *E.coli* isolates inhibited the pathogen strains, simultaneously. *E.coli* O157:H7 was inhibited chiefly by cow derived *E.coli* isolates (18.7%) as compared to sheep (12.5%) while from both sample equal number of colicin producing isolates inhibitory to O26:H11 were found.

**Table 1b.** Primers used for colicin, phylogenetic grouping and virulence genes, product sizes and PCR conditions applied.

| Genes | 5´-sequence-3´ | Length of PCR product | Temp conditions | | | Ref |
|---|---|---|---|---|---|---|
| | | | Danturation | Annealing | Extenstion | |
| colA | GTTGCGGAAAAAGCCAAAGA<br>CCCCAGAGCAACAGAGGAAG | 456 | 94°C 30s | 55°C 30s | 72°C 1min | Toshima *et al.*, 2007 |
| colB | AAGAAAATGACGAGAAGACG<br>GAAAGACCAAAGGCTATAAGG | 493 | | | | |
| colD | CTGGACTGCTGCTGGTGATA<br>GAAGGTGCGCCTACTACTGC | 420 | | | | |
| colE1 | GGTGGAACTGGAGGTAGCAA<br>CGTCGTTGTTCTGCTTCCTG | 356 | 94 °C 30s | 60°C 30s | 72°C 1min | Smajs *et al.*, 2010 |

| | | | | | | |
|---|---|---|---|---|---|---|
| colE2 | TGATGCTGCTGCAAAAGAG<br>TTCAAAGCGTTCCCTACCAC | 409 | | | | |
| colE3 | TAAGCAGGCTGCATTTGATG<br>TCGGATCTGGACCTTTCAAC | 413 | | | | |
| colE4 | GAAGGCTGCATTTGATGCT<br>CGGATCCGGACCTTTAATTT | 409 | | | | |
| colE5 | TAAGCAGGCTGCATTTGATG<br>TTGAATTCTCGAATCGTCCA | 430 | | | | |
| colE6 | ACCGAACGTCCAGGTGTT<br>TTTAGCCTGTCGCTCCTGAT | 399 | | | | |
| colE7 | GCATTCTGCCATCTGAAAT<br>CTTCTGCCCACTTTCTTTCG | 431 | | | | |
| colE8 | TAAGCAGGCTGCATTTGATG<br>GACTGATTGGCTTGTCGTGA | 449 | | | | |
| colE9 | TAAGCAGGCTGCATTTGATG<br>GACTTTTCTCCCTCCGACCT | 418 | | | | |
| colIa | TGTGGAAATTCACTGGGCGA<br>TCAGAAGAGCAGTGAGCGTG | 239 | 94°C 30s | 60°C 30s | 68°C 1min | Toshima *et al.*, 2007 |
| colIb | CAAATTCACTGGGCGAACGG<br>TCAGAAGAGCAGTGAGCGTG | 235 | | | | |
| colJs | CCGGACAACGGACAAAAACC<br>CATAACGCCAATGCTTCCCG | 478 | 94°C 30s | 55°C 30s | 72°C 1min | Smajs *et al.*, 2010 |
| colK | CAGAGGTCGCTGAACATGAA<br>TCCGCTAAATCCTGAGCAAT | 469 | | | | |
| colM | GCTTACCACTTCGCAAAACC<br>GAGCGACTCTCCGATAATGC | 429 | | | | |
| colS4 | TATATGGCCCAACTGCTGGT<br>CGTAAGGACGGACACCTGTT | 456 | | | | |
| col5 | CATTGGCAAAAGCGAAATCT<br>TGCAACTCTGGAAACAATCG | 443 | | | | |
| col10 | GGTTACCGGATTTCCTGGAT<br>TTCTAGATGCTTGGCCCACT | 448 | | | | |
| ChuA | GACGAACCAACGGTCAGGAT<br>TGCCGCCAGTACCAAAGACA | 279 | 94°C 30s | 55°C30s | 72°C 30s | Clermont *et al.*, 2000 |
| YjaA | TGAAGTGTCAGGAGACGCTG<br>ATGGAGAATGCGTTCCTCAAC | 211 | | | | |
| TspE4 C2 | GAGTAATGTCGGGGCATTCA<br>CGCGCCAACAAAGTATTACG | 152 | | | | |
| *stx1* | TGCCGGACACATAGAAGGAAACT<br>AGAGGGGATTTCGTACAACACTGG | 267 | 950C 30s | 48-550C 30s | 680C 30s | Tharmaraj *et al.*, 2009 |
| *stx2* | GGAGTTCAGTGGTAATACAATG<br>GCGTCATCGTATACACAGG | 149 | | | | |
| *hlyA* | GCTATGGGCCTGTTCTCCTCTGC<br>ACCACTTTCTTTCTCCCGACATCC | 224 | | | | |
| *ST1* | CTTTCCCCTCTTTTTAGTCAG<br>TAACATGGAGCACAGGCAGG | 175 | | | | |
| *eaeA* | GTGGCGAATACTGGCGAGACT<br>CCCCATTCTTTTTCACCGTCG | 891 | | | | |

*Colicin genes in inhibitory E.coli isolates*

Detection of colicin determinants showed that >80% colicinogenic *E.coli* retain multiple colicins (Figure 2). Colicin E6 was the most widely detected colicin with 52% *E.coli* isolates inhibiting *E.coli* O26:H11 while 31% inhibiting *E.coli* O157:H7. Colicin Ib was the second most prevalent colicin occurring in 70.8% *E.coli* isolates following E4, E7, J, Ia, M, S4 and E3 in 62.4%, 57%,41.6%, 31%, 29%, 22.8% and 18.7% *E.coli*, respectively. Moreover, col A, E5 and col10 producers only inhibited O26:H11 while col D and E1 only inhibited O157:H7 strains. Colicin E2 was detected in only one *E.coli* from cow sample inhibiting *E.coli* O157:H7 while remaining colicins B, K, E8, E9, and col 5 were not detected in any isolate.

**Table 2.** Phylogenetic groups, virulence genes and pathogenicity of isolated colicinogenic *E.coli*.

| *E.coli* isolates | Phylogenetic groups | Virulence genes | | | | | Pathogenicity |
|---|---|---|---|---|---|---|---|
| | | *Hly-α* | *stx1* | *stx2* | *st1* | *eaeA* | |
| C4 | D1 | - | - | - | - | - | NP |
| C6 | B1 | - | - | + | + | + | P |
| C7 | B1 | - | - | - | + | - | P |
| C19 | A0 | - | - | - | - | - | Np |
| C26 | D1 | - | - | - | - | - | NP |
| C29 | A0 | - | - | - | - | - | NP |
| C33 | B2 | - | - | - | - | - | NP |
| C34 | B2 | - | - | - | + | + | P |
| C40 | B2 | - | - | - | + | - | P |
| C52 | B2 | - | - | - | + | + | P |
| C53 | B1 | - | - | - | - | - | NP |
| C55 | D1 | - | - | - | - | - | NP |
| C59 | A1 | - | - | - | + | + | P |
| S4 | D1 | - | - | + | + | + | P |
| S6 | B1 | - | - | - | + | - | P |
| S7 | B2 | - | - | - | + | - | P |
| S10 | B1 | - | - | - | + | - | P |
| S19 | D1 | - | - | - | + | - | P |
| S20 | B2 | - | - | - | - | - | NP |
| S23 | D1 | - | - | - | - | - | NP |
| S25 | B2 | - | - | + | - | - | P |
| S29 | B2 | - | - | - | - | + | P |
| S44 | B2 | - | - | + | + | - | P |
| S54 | B1 | - | - | - | - | - | NP |
| S59 | B2 | - | - | + | - | - | P |
| H2 | A0 | - | - | - | - | - | NP |
| H6 | B2 | - | - | - | - | + | P |
| H8 | B1 | - | - | - | + | - | P |
| H15 | B2 | - | - | - | - | - | NP |
| H20 | B2 | - | - | + | + | - | P |
| H24 | D1 | - | - | - | - | - | NP |
| H37 | A1 | - | - | - | - | - | NP |
| H40 | A1 | - | - | - | + | + | P |
| H44 | B2 | - | + | - | - | - | P |
| H49 | B2 | - | + | - | - | - | P |
| H50 | B2 | - | + | + | + | - | P |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| H55 | B2 | - | + | - | + | - | P |
| H60 | B1 | - | - | - | - | - | NP |
| H62 | A1 | - | - | - | - | - | NP |
| H69 | D1 | - | - | - | + | + | P |
| H77 | B2 | - | - | - | + | - | P |
| H84 | B2 | - | + | + | + | - | P |
| H89 | A1 | - | - | - | + | - | P |
| H93 | B2 | - | + | - | - | - | P |
| H96 | B2 | - | - | - | - | + | P |
| H98 | B2 | - | - | - | - | - | NP |
| H105 | B2 | - | + | + | + | - | P |
| H113 | A0 | - | - | - | - | - | NP |

C= Cow, H= Human, S= Sheep, += detected, - = not detected, P=Pathogenic, NP= non- pathogenic.

*Phylogenetic groups and pathogenicity of colicinogenic E.coli*

According to scheme of *E.coli* phylogeny, it was observed that altogether group B2 *E.coli* was most prevalent i.e. 48%, however the frequency was highest in human specimen as compared to cow and sheep. The prevalence index of other groups was: 8.3% A0 *E.coli*, 10.4 % A1, 16.6% B1, and 16.6 % D1 *E.coli* strains (Table 2). No single isolate belonging to phylogroup D2 was detected among colicinogenic *E.coli* isolates.

Furthermore, detection of virulence genes in commensal *E.coli* showed that hemolysis gene (*hly-a)* was not found in any isolate which confirms the non-hemolytic nature of all *E.coli* isolates. *Stx*1 and *Stx*2 were found in 8.3% and 12.5% isolates, respectively while both genes occurred together in 6.25% isolates. *St*1 was detected in comparatively greater number of isolates i.e. 45.8%, whereas *eae*A was only found in 20.8% isolates.

**Table 3.** Antibiotic susceptibility profile of *E. coli* strains and their accession number as provided in NCBI database.

| *E.coli* No | Antibiotic resistance | *E.coli* strain | Accession No. |
|---|---|---|---|
| C19 | ND | *E.coli* W | CP002967.1 |
| C29 | ND | *E.coli* NRC129 | KP244268.1 |
| C53 | Amp, Step | *E.coli* W26 | AGIA00000000.1 |
| C55 | Amp, Step | *E.coli* ISO3 | KY971288.1 |
| S20 | Amp,Step | *E.coli* OZK1 | KT156725.1 |
| S23 | Amp, Tet | *E.coli* CNB12-2 | CP033635.1 |
| S54 | ND | *E.coli* CCFM8339 | KJ803896.1 |
| H2 | Ctx | *E.coli* W | CP002967.1 |
| H15 | Amp, Step | *E.coli* M160133 | CP022164.1 |
| H24 | Amp, Tet, Step,Ctx | *E.coli* ISO3 | KY971288.1 |
| H37 | Amp | *E.coli* NCTC86 | CP019778.1 |
| H60 | ND | *E.coli* CCFM8333 | KJ803890.1 |
| H62 | Cip, Step | *E.coli* Hs30-1 | CP029492.1 |
| H98 | Ctx, Step | *E.coli* s1428 | MG388227.1 |
| H113 | Amp, Step | *E.coli* NCTC11023 | LS483297.1 |

C= Cow, H= Human, S= Sheep, Amp= Ampicillin, Step= Streptomycin, Tet= Tetracylcine, Ctx=Cefotaxim, Cip= Ciprofloxacin, ND= not detected.

Almost 18 out of 48 isolates were considered as non-pathogenic since they were lacking all of the five virulence genes.

*Colicinogenic E.coli strains identified based on 16SrDNA sequence*

*E.coli* isolates (n=18) which were lacking all the given virulence genes were further sent to Macrogen, Korea, for strain identification on the basis of 16SrRNA sequencing. The results indicated that all the isolated *E.coli* strains were different from each other and were already previously reported in different studies. However, none of the strain showed 100% similarity index with the reported strains due to extensive rate of mutation in *E.coli* genome.

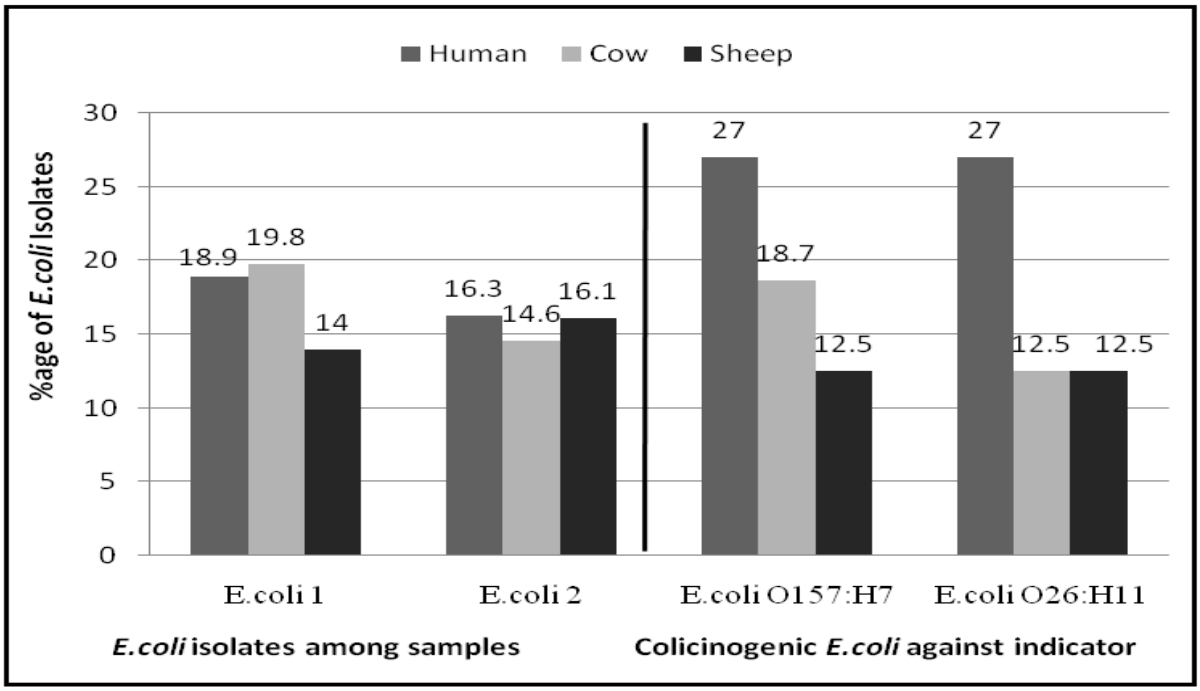


**Fig. 1.** Frequency of colicinogenic *E.coli* among different hosts.

The identified strains with their allotted accession numbers in the Nucleotide database are given in Table 3. Insight into the details of these strains produced in database confirmed that all the *E.coli* isolates belong to class of non-pathogens except three strains i.e. C4, C26 and C33 which possess some other virulence genes not tested in the given study. The pathogens were not further evaluated for their probiotic properties.

*Antibiotic resistance and iron scavenging habit of E.coli strains*

Identified non-pathogenic *E.coli* strains were evaluated for susceptibility to universally used antibiotics. Two cow (C19 and C24) and a sheep (S54) derived *E.coli* strains showed sensitivity to all antibiotics while among all six strains were resistant to ampicillin. Human strains H2, H62 and H98 were resistant to tetracycline, ciprofloxacin and cefotaxime, respectively. Strains H24 was eliminated from further characterization because of its multiple antibiotic resistances. Culturing *E.coli* strains on chrome azurol S agar plates showed few strains that produced slight a characteristic orange halo surrounding the colony. However, the results were considered insignificant for the given study which are not mentioned here.

**Discussion**

Commensal *E.coli* have potential role in inhibiting the accumulation of gut pathogen into the host (Rolf *et al.,* 2009). However, little information has been gathered regarding use of commensal microbial flora that can compete and inhibit the pathogenic species. The present study showed that 9.3% *E.coli* isolates

from cow, sheep and human were able to reduce the growth of two pathogens i.e. *E.coli* O157:H7 and *E.coli* O26:H11. Competitive exclusion of the target strains was achieved by release of protein antimicrobials (called colicins) which is one of the strategies attained by probiotic species to benefit host (DebRoy and Jayarao, 2002). Colicinogenic *E.coli* against O15:H7 from humans (27%) were greater in number, followed by cattle and sheep which correlates with previous observations where *E.coli* O157:H7 residing in cattle gut show resistance to indigenous colicins (Schamberger and Gonzalez, 2005). In a probiotic study by Zhao *et al.*, 1998, 18 out of total 1200 bacterial strains from cattle were inhibitory against *E.coli* O157:H7. Parallel results were shown by similar study where two *E.coli* isolates from cow and one from sheep, out of 112 colicinogenic *E.coli* inhibited the pathogen (Suresh *et al.*, 2014). *E.coli* O26:H11 was also inhibited greater number (27%) of human derived *E.coli*, in here, but almost equal number (12.5%) of *E.coli* isolates from cattle and sheep were found inhibitory towards it. *E.coli* O26:H11 and other Shiga toxin producing *E.coli* strains were killed by more than 15% of commensal *E.coli* isolated from feces of human, horses, pigs and sheep (DebRoy *et al.*, 2004), which correlate with the present study.

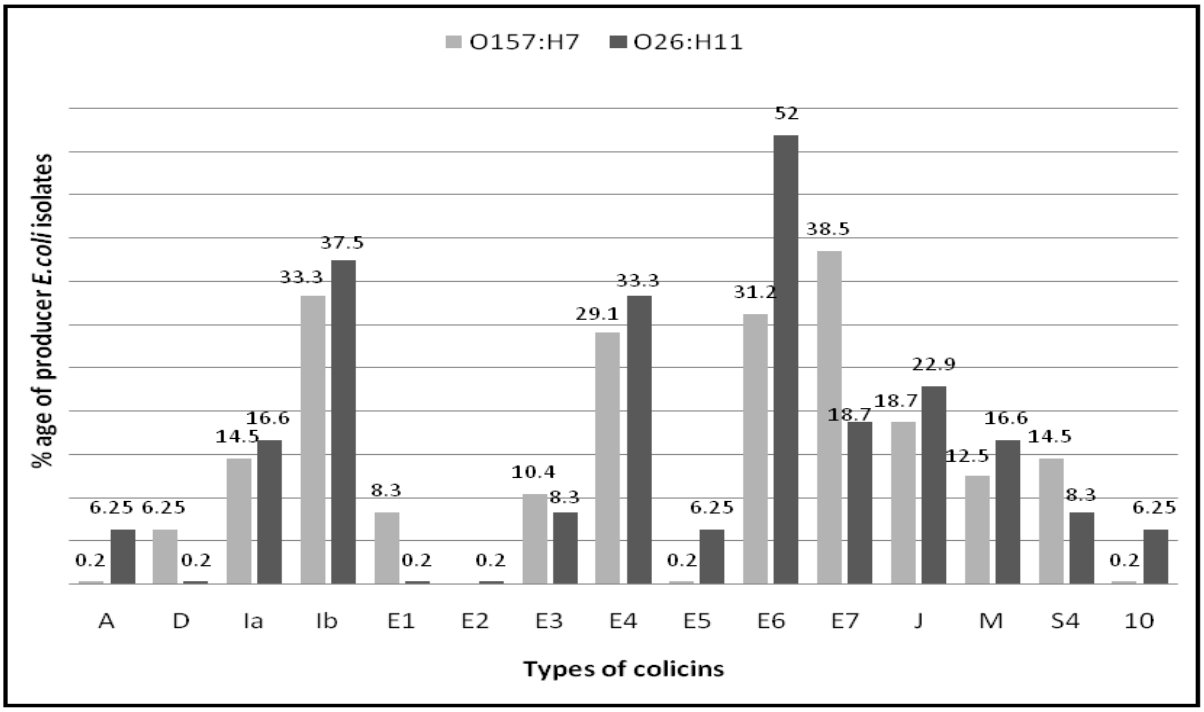


**Fig. 2.** Type of colicins found in colicinogenic *E. coli* inhibitory to pathogens.

*E.coli* strains produce multiple colicins with diverse mode of inhibition against the target species such as some are DNAses or RNAses while others are peptidoglycan inhibitor (Micenkova *et al.*, 2016). Molecular detection of colicins showed that almost all colicinogenic *E.coli* produced more than one colicin against each indicator strains. Colicin E6, E7 and colIb were enormously produced by these strains where col Ib is a pore forming while col E6 and E7 are DNases. Col E6 and Ib were extensively released against *E.coli* O26:H11while colE7 producers inhibited O157:H7 pathogen. Colicin Ia/Ib has been shown to reduce the growth of different strains of entero-hemorrhagic *E.coli* (Schamberger *et al.*, 2004). On the other hand, several research experiments have reported the anti-O157 ability of purified colE7 and colE2/E7 from different sources (Lobmann *et al.*, 2019).Col E6 and E7 were also simultaneously produced in >30% species inhibiting O157:H7. Several studies have reported the inhibitory action of purified colicins B, E1, E2, E7, K, and Ia/Ib against *E.coli* O157: H7, however, extent of inhibition

and the dose of colicins has not been defined yet (Bradley *et al.*,1990, Murinda *et al.*, 1996, Schamberger *et al.*, 2002). Other colicins such as A, D, Ia, J, M, S4, E1, E3 and E5 were also found produced by *E.coli* isolates inhibitory towards entero-hemorrhagic pathogens; however, few previous studies have confirmed that inhibition mode of these colicins against the given pathogens. Col Ia, J, M, E3 and S4 were produced by almost equal number of *E.coli* isolates against both pathogens. Coilcin A which is rarely reported in commensal species (Micenkova *et al.*, 2014) was produced in 7% species inhibitory against *E.coli* O157:H7 while colicin D produced by only human derived *E.coli* was active against O26:H11. Similarly, col E1 and col 10 were inhibitory towards each of the pathogens i.e. O157:H7 and O26:H11, respectively. Production of multiple colicins at a time against the given pathogen indicates the strong competition of *E.coli* towards competitorspecies.

Characterization of colicinogenic *E.coli* on the basis of phylogenetic groups and virulence genes indicated that indicated that pathogenicity was more prevalent among phylogroup B2 (39.5%) as compared to all other groups, followed by 10.4% B1, 6.2% D1 and 6.2% A1 group. Phylogroup A0 isolates were all lacking virulence genes. Increased incidence of B2 group among commensal *E.coli* in the given study is contradictory to previous reports (Kohoutova *et al.*, 2014), which suggests that there is a drastic change of microbial diversity over period of time and it involves various factors i.e. exchange of genes between invading and indigenous microflora. However, others have reported B2 *E.coli* as retaining diverse colicin and virulence genes (Willyard, 2017). According to DebRoy and Madydox (2001), *E.coli* strains considered as probiotics should be screened for several virulence genes before being commercialized since it can cause horizontal gene transfer into normal flora of the host. For the purpose, five virulence genes were used as representative of all groups of enteropathogenic *E.coli (hlya, St, Stx1, Stx2 and eaeA).* An experiment designed to investigate pathogenicity in cattle *E.coli* showed that *eae*A was commonly found in 22.11% species, where >50% species contained *eae*A together with *stx*2 and *st* (Camila *et al.*, 2010).

These results correlates with the present study where *st* and *eae*A were detected in 45% and 20% *E.coli* isolates, respectively. Recently, the association of phylogenetic groups with the presence of virulence genes were eliminated (Camila *et al.*, 2010) which confirms the given observations where four B2 *E.coli* did not possess all of the virulence genes. Colicinogenic *E.coli* (n=15) were confirmed as non-pathogenic strains on the basis of 16srDNA sequencing (Table 3) while remaining species were not further tested. Antibiotic resistance profiling and siderophore assay of the all 15*E.coli* strains showed that *E.coli* strains S54 (*E.coli* CCFM8339) and H60 (*E.coli* CCFM8333) possesses fitness factors that have strong competitive effect on enterohemorrhagic *E. coli* O157:H7 and enterotoxigenic *E. coli* O26:H11. Moreover, they lack important virulence factors associated with various pathovars of *E.coli.* Release of multiple colicins (i.e. col Ib, E4,E6, E7 by S54 and col Ia, Ib, J , E3, E4, E6, E7 by H60) and sensitivity to antibiotics permit the use of these strains as effective probiotics in livestock and for humans as well. *In-vivo* animal trials will be further designed to thoroughly investigate probiotic potential of these strains.

**Conclusion**

Our results showed that commensal *E.coli* strains that retain multiple colicin determinants can effectively inhibit the growth of sensitive pathogenic *E. coli.* Absence of virulence genes, sensitivity to antibiotics, non-hemolytic property and non-invasive properties makes these strains potential probiotics to be used as therapeutic agents against given pathogens in humans and animals both.

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