

Phylogenetic Grouping and Phenotypic Detection of Extended-Spectrum β -Lactamases Among *Escherichia coli* From Calves and Dairy Cows in Khuzestan, Iran



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Abstract

Background: Food-producing animals are under suspicion for the reservoir and colonization of ESBL (extended-spectrum beta-lactamase)-producing bacteria especially Enterobacteriaceae and therefore infection of the humans with them. The increasing reports on the ESBLs presence in the pathogenic and commensal *Escherichia coli* isolates have been a concern worldwide. These strains can be attributed to one of the main phylogenetic groups and subgroups. Several studies have shown the relationship between the phylogeny and antimicrobial resistance of *E. coli* strains.

Objectives: The aim of this study was to analyze the phylogenetic group of ESBL-producing *E. coli* and detect its phenotype using the multiplex polymerase chain reaction (PCR) and combined disk method.

Materials and Methods: Two hundred five *E. coli* fecal isolates were obtained from 103 calves (90 healthy and 13 diarrheic) and 102 dairy cows (healthy) from 8 farms in Khuzestan province, Iran. The triplex PCR method was used to allocate the *E. coli* isolates based on the presence or absence of 3 genes (*chuA*, *yjaA*, and *tspE4.C2*) to yield 4 definite phylogenetic groups and 7 subgroups. Phenotypic ESBL-producing *E. coli* was determined using the double disk diffusion method according to the manufacturer's instructions and Clinical & Laboratory Standards Institute (CLSI) guidelines.

Results: A total of 65.04% and 22.3% of isolates from calves and 70.5% and 20.5% of isolates from dairy cows belonged to phylogroups B1 and A, respectively. In addition, no isolate from the diarrhoeic calves was found to belong to group B2 and subgroups D2 and A0. A low prevalence (2/205 isolates, 0.97%) of ESBL-producing *E. coli* was found only in the samples of dairy cows which belonged to the phylogenetic group A and phylogenetic subgroup A1. There was no statistically significant relationship between the phylogenetic group and the production of ESBLs ($P=0.11$). There was also no difference between the *E. coli* isolates from calves and dairy cows in the production of ESBLs ($P=0.5$).

Conclusion: There was no statistically significant relationship between the phylogenetic group and the production of ESBLs ($P=0.11$). There was also no difference between *E. coli* isolates from calves and dairy cows in the production of ESBLs ($P=0.5$). Based on these results, there is a low prevalence of ESBL-producing *E. coli* in the dairy farms of Khuzestan province. However, further large scale investigations are necessary to control the antibiotic resistance in the human and animal foodstuff.

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Background

Escherichia coli strains are the major members of Enterobacteriaceae which are usually found in the digestive system of human and warm-blooded animals. Certain *E. coli* strains have been associated with neonatal diarrhea and extraintestinal infections in ruminants which cause appreciable economic losses in dairy industry all around the world.^{1,2} Phylogenetic analyses have shown that *E. coli* strains are located in 4 main phylogenetic groups (A, B1, B2, and D) and the strains differ in phenotypic characteristics including antibiotic-resistance patterns, and resistance to special antibiotics can be related to particular phylogenetic groups.³⁻⁶ The World Health Organization (WHO) reported that approximately 50% of the universally produced antibiotics are consumed for veterinary usages such as prophylactic and growth promotion purposes.⁷⁻⁹ The increase of antibiotic resistance following the use of antibiotics such as beta-lactams and growing the commensal and pathogenic ESBL (extended-spectrum beta-lactamase)-producing bacteria like *E. coli* strains especially in food-producing animals pose a threat to the public health and food safety.¹⁰⁻¹⁴ Researchers have suggested that the study of antibiotic resistance in commensal microorganisms like *E. coli* from the healthy animals would be quite useful, because they can be used as gnomon organisms to measure the prevalence of antibiotic resistance. These organisms could serve as a reservoir for the genes encoding antimicrobial resistance, which is transferred to the pathogenic bacteria or transmitted to the human by direct exposure to animals or via the food chain.¹⁵⁻¹⁹ It has been shown that the resistant bacteria with animal origin can either colonize or cause infection in humans.²⁰

Objectives

Due to the importance and spread of ESBL-producing *E. coli* strains in the food-producing animals and possible relationship between the ESBL production and phylogenetic groups, the aim of this study was to evaluate the prevalence of ESBL-producing bacteria and the phylogenetic groups, on *E. coli* fecal isolates from calves and cows in dairy farms of Khuzestan province.

Materials and Methods

Sample Collection

A total of 237 fecal samples from healthy dairy cows (134 samples), healthy (90 samples) and diarrheic (13 samples) calves were aseptically collected from 8 dairy farms located in Khuzestan province, Iran. Samples were randomly obtained from the animals by recto-anal mucosal swab (RAMS) method and transported on ice to the microbiology laboratory. The samples were cultured on Mac Conkey agar and incubated at 37°C for 24 hours. Three to 5 dark pink colonies (presumptive *E. coli*) were randomly selected and identified by subculturing on eosin methylene blue plate (EMB) and confirmed to be *E. coli* using the standard biochemical tests.^{21,22} The confirmed *E. coli* isolates were stored in skim milk at -70°C for further

experiments.

DNA Extraction

DNA extraction from isolates was performed by boiling method as described previously.²³ Briefly, a few colonies were suspended in 300 µL sterile distilled water and heated at 95°C for 10 minutes. Afterward, they were put on ice for 5 minutes. Then, they were centrifuged at 13 000 rpm for 10 minutes and the supernatant was used as the DNA template.

Phylogenetic Grouping by Multiplex Polymerase Chain Reaction

The triplex polymerase chain reaction (PCR) method was used to allocate the *E. coli* isolates based on the presence or absence of 3 genes (*chuA*, *yjaA*, and *tspE4.C2*) to yield 4 distinct phylogenetic groups (A, B1, B2, and D) and 7 subgroups (A0, A1, B1, B2₂, B2₃, D1, and D2) (Table 1).^{6,24} Multiplex PCR was achieved in a 25 µL reaction mixture, including master mix (12.5 µL, containing Tris-HCl pH 8.5, (NH₄)₂S₀₄, 2mM MgCl₂, 0.2% Tween 20, 0.4mM dNTPs, 0.2 units/µL Ampliqon Taq DNA polymerase and inert red dye) (Denmark), each primer (1 µL from 10µM) (Bioneer, South Korea), Distilled water (2.5 µL), and template DNA (4 µL). The primer pairs used for PCR amplification are shown in Table 2.⁶ A reaction with DNA of ECOR62 having *chuA*, *yjaA*, and *TspE4.C2* and a reaction without the template DNA was used as positive and negative controls, respectively. The PCR program was performed in a thermal cycler (Eppendorf, Germany) as follows: initial denaturation at 94°C for 5 minutes, 30 cycles of the 30 seconds at 94°C, 30 seconds at 55°C and 30 seconds at 72°C, followed by a final extension at 72°C for 7 minutes.⁶ Amplification products were separated on 1.5% agarose gel containing safe stain (Cinnagen, Iran).

Phenotypic Detection of ESBL in *Escherichia coli* Isolates

An initial screen test was done using ceftriaxone (30 µg) disk (Mast diagnostic, UK) with the Kirby-Bauer method advised by NCCLS (formerly known as the National Committee for Clinical Laboratory Standards). On the initial screen test, the isolates that showed a zone of inhibition ≤25 mm were then confirmed for ESBL production by the phenotypic confirmatory test. The

Table 1. Combination of *chuA*, *yjaA*, and *TspE4.C2* Genes for Determination of Phylogenetic Subgroups of *Escherichia coli*²²

Phylogenetic Subgroups	Genes Combination Profile
A0	(<i>chuA</i> -, <i>yjaA</i> -, <i>TspE4.C2</i> -)
A1	(<i>chuA</i> -, <i>yjaA</i> +, <i>TspE4.C2</i> -)
B1	(<i>chuA</i> -, <i>yjaA</i> -, <i>TspE4.C2</i> +))
B22	(<i>chuA</i> +, <i>yjaA</i> +, <i>TspE4.C2</i> -)
B23	(<i>chuA</i> +, <i>yjaA</i> +, <i>TspE4.C2</i> +))
D1	(<i>chuA</i> +, <i>yjaA</i> -, <i>TspE4.C2</i> -)
D2	(<i>chuA</i> +, <i>yjaA</i> -, <i>TspE4.C2</i> +))
Untypeable	(<i>chuA</i> -, <i>yjaA</i> +, <i>TspE4.C2</i> +))

Table 2. The Primer Sequence of Genes for Determination of *Escherichia coli* Phylogenetic Groups⁶

Primer name	Sequence (5'-3')	Target Gene	Product Size (bp)
<i>ChuA</i> (F)	GAC GAA CCA ACG GTC AGG AT	<i>chuA</i>	279
<i>ChuA</i> (R)	TGC CGC CAG TAC CAA AGA CA	<i>chuA</i>	279
<i>YjaA</i> (F)	TGAAGTGTCCAGGACGCTG	<i>yjaA</i>	211
<i>YjaA</i> (R)	ATG GAG AAT GCG TTC CTC AAC	<i>yjaA</i>	211
<i>TspE4.c2</i> (F)	GAG TAA TGT CCG GGC ATT CA	<i>TspE4.c2</i>	152
<i>TspE4.c2</i> (R)	CGC GCC AAC AAA GTA TTA CG	<i>TspE4.c2</i>	152

ESBL phenotypic confirmatory test was determined by the double disk diffusion method including cefotaxime (30 µg)/cefotaxime-clavulanic acid (30 µg/10 µg) and ceftazidim (30 µg) /ceftazidime-clavulanic acid (30 µg/10 µg) (Mast Diagnostic, UK) on Mueller-Hinton agar (Merck, Germany) according to the manufacturer's instructions and Clinical & Laboratory Standards Institute (CLSI) guidelines. The strains of *E. coli* (ATCC 25922) and *Klebsiella pneumoniae* (ATCC 700603) were respectively used as negative and positive controls in this study. The isolates were reported as ESBL-producing *E. coli* when a zone with increased diameter (≥ 5 -mm) was observed for antimicrobial agent tested in combination with clavulanic acid compared to the zone diameter of the agent when tested alone.²⁵

Statistical Analysis

Analyses of data were performed by SPSS software (version 16.0) and proportions were compared using the Fisher exact tests with the significance level set at $P < 0.05$.

Results

Out of a total of 237 fecal samples, 205 *E. coli* isolates were obtained from the calves and dairy cows and confirmed.

Phylogenetic grouping analyses showed that *E. coli* isolates were distributed among groups B1, A, D, and B2

with 67.8%, 21.4%, 6.8%, and 1.9% prevalence indexes, respectively. All phylogenetic groups and subgroups were observed among isolates but there was no isolate from the diarrheic calves belonging to the B2 group and A0 and D2 phylogenetic subgroups (Table 3). Furthermore, 4 isolates were remained untypable by Clermont method (Figure 1).

The results of initial screen test for the phenotypic detection of ESBL-producing *E. coli* strains showed that 35 isolates (20 isolates from cows and 15 isolates from calves) were ceftriaxone resistant. By the ESBL phenotypic confirmatory test, only 2 isolates from the dairy cows were confirmed for ESBL-producing *E. coli* (Table 4). All ESBL-producing isolates were found to belong to the phylogenetic group and subgroup A1. There was no statistically significant relationship between the phylogenetic group and the production of ESBLs ($P=0.11$) and also no difference was observed between the calves and cows in the production of ESBLs ($P=0.5$).

Discussion

Commensal *E. coli* derived from farm animals have genetic diversity and tracking them is of importance, as they are considered as hidden sources of resistant and virulent genes.²⁶⁻²⁹ The increasing of pathogenic and commensal ESBL-producing *E. coli* strains are originated from different sources (such as human, food, and animal)

Table 3. Distribution of the *Escherichia coli* Phylogenetic Subgroups in Studied Animals

Phylogenetic Subgroups	Healthy Calves	Diarrheic Calves	Dairy Cows
A ₀	7 (7.7%)	-	4 (3.9%)
A ₁	14 (15.5%)	2 (15.3%)	17 (16.6%)
B1	59 (65.5%)	8 (61.5%)	72 (70.5%)
B2 ₂	1 (1.1%)	0 (0%)	1 (0.98%)
B2 ₃	1 (1.1%)	0 (0%)	1 (0.98%)
D ₁	5 (5.5%)	2 (15.3%)	2 (1.96%)
D ₂	1 (1.1%)	0 (0%)	4 (3.9%)
Total	88 ^a	12 ^a	101 ^a

^aFour strains were untypable.

Table 4. ESBL Pattern of *Escherichia coli* Isolated From Fecal Specimens of Calves and Dairy Cows

Animals	No. of Collected RAMS Samples	No. of <i>E. coli</i> Isolates	No. (%) of Ceftriaxone Resistant Isolates	No. of Confirmed ESBL-Producing <i>E. coli</i>
Calves	103	103	15 (14.5)	0(0)
Dairy cows	134	102	20 (19.6)	2(1.9)
Total	237	205	35 (17)	2(0.97)

Abbreviations: RAMS, recto-anal mucosal swab; ESBL, Extended-Spectrum β -Lactamase.

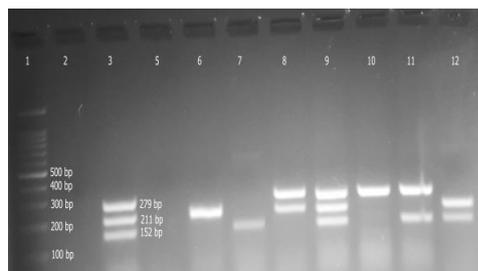


Figure 1. Triplex PCR Profiles Specific for *Escherichia coli* Phylogenetic Groups and Subgroups.

Each combination of amplified *chuA* (279 bp), *yjaA* (211 bp), and *TSPE4.C2* (152 bp) genes allowed the phylogenetic group and subgroup determination of a strain. Lane 1: Marker 100 bp (SinaClon); lane 2: negative control; lane 3: positive control; lane 4: phylogenetic group A (A0); lane 5: phylogenetic group A (A1); lane 6: phylogenetic group B1; lanes 7–8: phylogenetic group B2 (B₂₂ and B₂₃); lanes 9–10: phylogenetic group D (D1 and D2); and lane 11: untypable.

and the dissemination of antibiotic resistance among other members of Enterobacteriaceae is a worldwide problem in the control and treatment of infections in human and animals.^{20,30-34} Moreover, some studies have shown that infections caused by ESBL-producing bacteria leads to the increase in the economic costs of infections treatment and the morbidity and mortality of diseases.³⁵ Furthermore, resistance to other antibiotic classes was observed in ESBL-producing *E. coli* more than non-ESBL-producing isolates.^{31,36} It has been shown that the intestinal pathogenic *E. coli* strains mainly belong to the phylogenetic groups A, B1, and D and the commensal strains belong to the groups A and B1, while extraintestinal pathogenic strains usually belong to the groups B2 and D. In our study, most of the isolates were found to belong to the phylogenetic groups B1 and A (A1). These results were almost similar to other studies which demonstrated that the isolates of phylogroup A were prevalent in the intestinal tracts of most animals^{24,37} and phylogroup B1 was dominant among isolates from healthy cattle.³⁸

In accordance with the previous reports in Iran,^{23,39} this study showed that the phylogroup B1 was dominant in calves. The phylogroup B2 had also the least frequency, both in calves and dairy cows, and these outcomes confirmed the previous investigations.²³ In contrast to a previous study¹ that reported subgroup B₂₃ as only present in the human and B2 especially B₂₃ as a good index of human fecal pollution, our results showed that subgroup B₂₃ was found among isolates from both calves (healthy) and dairy cows. Similarly, another study showed that *E. coli* isolates of ruminants (sheep, goat, calves, and cattle) and dogs belonged to the subgroup B₂₃.^{23,40} Phylogenetic subgroups D2 and A0 and phylogroup B2 were not found among isolates from diarrheic calves and our results were similar to previous researches which showed that phylogenetic subgroups D2 and A0 and phylogroup B1 were not found among isolates from diarrhoeic calves.²³ Though difficult to claim, the geographic variation, ecological conditions, environmental and health statuses

play a significant role in *E. coli* phylogroup dispersion. Additionally, the prevalence of phylogenetic groups in animals is dependent on the body volume and host food diet.⁴¹

There are some reports on the ESBL-producing *E. coli* in sheep and poultry in Iran,⁴²⁻⁴⁴ however based on a search in the databases we could not find any data about ESBL-producing *E. coli* in calves and dairy cows in Khuzestan province. In this study, the prevalence of ESBL-producing *E. coli* and their association with the phylogroups of *E. coli* isolates from calves and dairy cows were considered. First, the ESBL-producing isolates were determined by the combined disk method and by PCR, and then the plenty and distribution of phylogenetic groups in *E. coli* isolates were investigated. Afterward, the relative frequencies of four phylogenetic groups of ESBL-producing *E. coli* populations were compared. A low prevalence (2/205 isolates, 0.97%) of ESBL-producing *E. coli* was found only in the cow samples which belonged to the phylogenetic group A and phylogenetic subgroup A1. Our results were in accordance with the results of Valat et al⁴⁵ who reported that ESBL-producing *E. coli* isolates from cattle belonged mainly to the phylogroup A. The low prevalence of ESBL-producing *E. coli* was similar to the results of Shiraki et al,⁴⁶ but it was very lower than that of some previous studies.⁴⁷⁻⁵²

Our results showed that there was no statistically significant relationship between the phylogenetic group and the production of ESBLs ($P=0.11$). Previous studies showed that the use of antibiotics may strongly influence the phylogenetic group distribution of *E. coli* isolates.⁵³ The resistance to different antibiotics was also particularly common for the phylogroups A and D and decreased in other groups.⁵⁴ It is believed that alteration in antimicrobial agents' resistance occurs more easily in the phylogenetic groups of *E. coli* strains except for B₂.⁵⁴ Such communication between the phylogenetic groups and the antibiotic resistance of strain could describe why farm mammals undergo antibiotic pressure. The strains of phylogenetic groups of A and B1 are selected and B2 strains counter-selected.

In conclusion, food-producing animals are under suspicion for the reservoir and colonization of ESBL-producing bacteria especially Enterobacteriaceae and therefore infection of the humans with them. Further large scale investigations are necessary to control the antibiotic resistance in the human and animal foodstuff.

Authors' Contributions

Experiment design, experiment conduct, data interpretation and manuscript edition by DGH and MGH; sampling and study design by MHH; statistical analysis by MPB; literature review, laboratory performance and practice by MB.

Conflict of Interest Disclosures

The authors have declared that no conflict of interests exists.

Ethical Approval

We hereby declare that all ethical standards have been respected in the preparation of the article.

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