Comparative Immunology, Microbiology and Infectious Diseases 63 (2019) 117–126

Contents lists available at ScienceDirect

Comparative Immunology, Microbiology and Infectious Diseases

journal homepage: www.elsevier.com/locate/cimid

# Antibiotic resistance, ESBL genes, integrons, phylogenetic groups and MLVA profiles of *Escherichia coli* pathotypes isolated from patients with diarrhea and farm animals in south-east of Iran

Roholla Taghadosi[a], Mohammad Reza Shakibaie[b,*], Hossein Hosseini -Nave[a]

[a] Department of Microbiology and Virology, Kerman University of Medical Sciences, Kerman, Iran
[b] Infection Diseases and Tropical Medicine Research Center, Kerman university of Medical Sciences, Kerman, Iran

ARTICLE INFO

*Keywords:*
*Escherichia coli*
Antibiotic resistance
Extended-spectrum β-lactamases
Phylogenetic group
MLVA

ABSTRACT

The aims of this study were to investigate the prevalence, antibiotic resistance, presence of class 1 and 2 integrons, Extended Spectrum β-Lactamases (ESBL) genes, phylogenetic group and epidemiological relationships of EPEC, ETEC and EHEC pathotypes isolated from patients with diarrhea and farm animals in south east region of Iran. A total of 671 diarrheagenic *E. coli* (DEC) were collected from stool samples of 395 patients with diarrhea and 276 farm cattles and goats. Presence of EPEC, ETEC and EHEC were identified using multiplex-PCR employing primers targeted the shiga toxin (*stx*), intimin (*eae*), bundle forming pili (*bfp*), and enterotoxins (*lt* and *st*) genes. The highest proportion of the patients (64%) were children under age 1–15 year ($p \leq 0.05$). Among the isolates, atypical EPEC was detected in 26 patients and 14 animal stool samples, while typical EPEC was found in 2 cattles. ETEC isolates were detected in stools of 13 patients and 4 EHEC was identified in 3 goats and one cattle. The isolates were checked for susceptibility to 14 antibiotics. 50% (n = 13) of EPEC and 61.5% (n = 8) of ETEC showed multi-drug resistance (MDR) profiles and one EPEC was found to be extensive drug resistant (XDR). In contrast, EHEC isolates were susceptible to the majority of antimicrobial agents. The MDR isolates were positive for $bla_{TEM}$ and $bla_{CTX-M}$ ESBL genes and carried class 1 integrons. Further study on the biofilm formation indicated that, 3 out of 4 EHEC isolates showed strong biofilm, while other pathotypes had either moderate, weak or no biofilm activity. Majority of EPEC isolates were belonged to phylogenetic group B1, all except one ETEC were classified as phylogenetic group A and two EHEC were belonged to phylogroup D, respectively. A multilocus variable tandem repeat analysis (MLVA) exhibited 22 distinct patterns. In conclusion, MLVA data showed high clonal diversity. Presence of EHEC in animal origins pose public health concern in this region.

## 1. Introduction

One of the most important bacterial agents which causes diarrhea in developing countries is diarrheagenic *E. coli* (DEC) [1]. There are six DEC pathotypes including enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC), enterohemoragic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), and diffusely adherence *E. coli* (DAEC) [2].

EPEC is a major etiological agent of infant diarrhea predominantly in developing countries [3]. This pathotype subdivided into typical and atypical strains based on plasmid-mediated bundle forming pilus (*bfpA*), and the *eae* gene [4,5]. The prevalence of atypical EPEC is higher than typical EPEC in several countries [6] and are present in both healthy and diseased animals (cattle, goat, cat and dog) and humans [7].

ETEC is the leading cause of traveler's diarrhea of adults in industrialized countries and children in developing countries. These strains carried two enterotoxins encoded by *lt* and *st* genes and a colonization factor [8]. The genes encoding the enterotoxins and the colonization factor are located on plasmids found across diverse ETEC serogroups. Whole genome sequencing of a representative collection of ETEC isolated between 1980 and 2011 identified globally distributed lineages characterized by distinct colonization factor and enterotoxin profiles [9].

EHEC is the most virulent pathotype of *E. coli* associated with severe infections such as hemorrhagic colitis and hemolytic uremic syndrome (HUS) in industrialized countries [10]. The strains produce shiga like toxin encoded by *stx* gene [11]. Cattle, sheep, goat and other farm

* Corresponding author.
*E-mail address:* mr_shakibaei@kmu.ac.ir (M.R. Shakibaie).

https://doi.org/10.1016/j.cimid.2019.01.004
Received 22 July 2018; Received in revised form 3 January 2019; Accepted 9 January 2019

animals are a major reservoir of EHEC, but unlike in humans, EHEC colonization in adult ruminants is asymptomatic [11].

Microbial biofilms are structurally and dynamically complex biological systems comprising populations of microorganisms that are concentrated at an interface (usually solid–liquid) and typically surrounded by an extracellular polymeric substance (EPS) matrix [12]. Biofilms also constitute a distinct growth phase that is clearly different from its planktonic counterpart [13]. The bacteria in biofilms are metabolically less active, resistant to environmental stress and engage in more genetic exchange [14,15]. Hospital isolates of *E. coli* often attach to hospital instruments via biofilm formation and are resistance to different classes of antibiotics. In contrast, there is no link between ability to form a strong biofilm and antibiotic resistances in community acquired infections of *E. coli* pathotypes EPEC, ETEC and EHEC [14].

Molecular typing methods such as MLVA and PFGE are frequently applied for accessing genetic relatedness among bacterial pathogens for the purpose of epidemiological surveillance. MLVA is a PCR-based method used for distinguishing bacterial lineage same or different species by targeting specific variable number tandom repeat (VNTR) loci [16]. MLVA exploits the variations in the copy number at 7 or more tandem repeat loci allowing the grading of isolates relatedness. This method has already been demonstrated more discriminatory than other typing methods [16].

In previous works, we studied virulence genes, phylogenetic groups and biofilm formation among STEC and EAEC pathotypes in south east Iran [17,18]. The aims of this study were to investigate and compare the antibiotic resistance, presence of class 1 and 2 integrons, existence of ESBL genes, phylogroup analysis, biofilm formation and MLVA profiles of EPEC, ETEC and EHEC isolates recovered from patients with diarrhea and farm animals (cattles and goats) in south east Iran.

## 2. Materials and methods

### 2.1. Bacterial sampling and identification

A total of 671 *E. coli* were collected from rectal samples of 395 patients with diarrhea and 276 healthy cattles and goats from 5 dairy and beef farms located near Kerman city, south east of Iran. Animal samples were collected by a veterinarian from October 2014 through November 2015. The minimum animal sample size required for detecting a difference with a 95% confidence level at 5% was estimated as n = 276. The sample size was determined on the basis of average goat and cow population size of five organized and unorganized dairy and beef farms in Kerman city, south-east of Iran (Fig. 1). The samples were collected using simple random sampling method. The organized farms in this study included farms maintained by private section and farmers by stall feeding, while, unorganized farms included goats reared as free grazing, where mixed farming was commonly practiced. The average ages of the goats and cattles were classified into groups of one year and > one-year-old. Please see the Supplement 1.

The specimens inoculated into 5 mL sterile transport Stuart medium (Merck, Darmstadt, Germany) and transferred to microbiology laboratory, KMU University for further investigations. In case of Human, the diarrheic patients referred to 2 main hospitals (Afzalipuor and Payambar-Azam) were enrolled in this study if they had diarrhea characterized by frequent watery stools (> 3 times/day) with or without blood or mucus. Those patients that were under treatment with antibiotics were omitted from this study. All were outpatients and referred to the above hospitals from local area (Supplement 1). Duplicate specimens were collected by means of sterile cotton swabs applied to the rectums of case patients by expert technician, checked microscopically for presence of any parasite or cysts and broth to our laboratory for further investigations. All stool samples were cultured on the surfaces of MacConkey and Eosin Methylene Blue (EMB) agar plates, incubated at 37 $^0$C for 24 h. Lactose-positive colonies were then re-cultured with the standard biochemical and conventional diagnostic tests for detection of *E. coli* as described previously [19].

### 2.2. Ethical approval

This study was approved by the Ethics Committee of the Kerman University of Medical Sciences, Kerman provenience, Gov. of Iran.

### 2.3. Detection of E. coli pathotypes by PCR

The EHEC, EPEC and ETEC pathotypes were detected by Multiplex-polymerase chain reaction (Multiplex-PCR) method using specific primers targeted the shiga toxin (*stx)*, intimin (*eae)*, bundle forming pili *(bfp),* and enterotoxins *lt* and *st* genes, respectively. Briefly, we removed five

*E. coli* colonies plated on MacConkey agar, mixed with 200 µL of double-distilled water in 1.5 mL DNase free sterile microcentrifuge tubes (Eppendorf, Germany) and boiled for 10 min in a water bath followed by snap chilling in ice for 5 min. The heat-treated bacterial suspensions were centrifuged at 10.000 rpm for 7 min to pellet down the cell debris, and the supernatants were used as DNA templates in the PCR assays [20]. The primers (Macrogen, Seoul, Korea) and annealing temperatures are illustrated in Table 1. The amplification procedure and electrophoresis of PCR products were carried out according to the method described previously [21–23]. Here, we used three reference strains EPEC E3204/2 (*eae* + *bfp*), EHEC E9812/22 (*stx* + *eae*), STEC EDL955/6 (*lt* + *st*) as positive control for PCR assays. The strains were kindly obtained from Pasture Institute of Iran. All the isolates were stored at −70 °C in Trypticase Soy Broth (Difco Laboratories, Detroit, Mich.) containing 30% glycerol for further study.

### 2.4. Antibiotic susceptibility and ESBL detection

Susceptibility of isolates to 14 antibiotics was determined by disc diffusion break point assay in accordance with the guidelines recommended by Clinical and Laboratory Standards Institute (CLSI 2016) [24]. The antibiotic discs were purchased from Mast Group Ltd UK, and used with following concentrations; Aztreonam (ATM: 30 µg), Amikacin (AK: 30 µg). Piperacillin (PRL:

100 µg), Augmentin (AUG: 30 µg), Kanamycin (K: 30 µg), Ciprofloxacin (Cip: 5 µg), Ceftriaxone (CRO: 30 µg), Ceftazidime (CAZ: 30 µg), Cefotaxime (CTX: 30 µg), Tobramycin (TN: 10 µg), Nalidixic acid (NA: 30 µg), Trimethoprim-sulfamethoxazole (TS: 25 µg), Ampicillin (Ap: 10 µg), Imipenem (IMP: 10 µg). The zone diameter was used for the interpretative results “susceptible” (S), “intermediate” (I), or “resistant” (R) against the tested antibiotic as described by manufacturer. *E. coli* ATCC25922 was used as control strain.

Detection of ESBL enzymes was carried out by double disc synergy test (DDST) [25]. Briefly, overnight grown culture suspension of the 57 strains EHEC, EPEC and ETEC pathotypes adjusted to $1 \times 10^8$ CFU/mL (0.5 McFarland standard) by sterile D/W and inoculated on the lawn surface of Mueller- Hinton agar plates (Merck, Germany). The cefotaxime (30 µg) / cefotaxime + clavulanic acid (30 µg/10 µg), and the cefpodoxime (30 µg) / cefpodoxime + clavulanic acid (30 µg/10 µg) discs were then placed 20 mm apart on the agar. After incubating overnight at 37 °C for 24 h, a ≥ 5 mm or more increase in the zone diameter around cefotaxime/clavulanic acid or cefpodoxime / clavulanic acid was considered positive for ESBL production. In this experiment, *Klebsiella pneumoniae* ATCC 700603 was used as quality control strain.

For detection of ESBL genes, we inoculated single colony of each *E. coli* isolate into 5 mL of Luria Bertani broth (LB) and incubated for 24 h at 37 $^0$C. Cells were centrifuged at 10.000 rpm for 5 min and precipitates were dissolved into 400 µL Tris-EDTA buffer (pH-8.0), subjected to boiling for 10 min and centrifuged. Supernatant was then used as template DNA for PCR reaction. Here, we selected primers for amplifications of three most prevalent ESBL genes ($bla_{\text{CTX-M}}$, $bla_{\text{TEM}}$ and

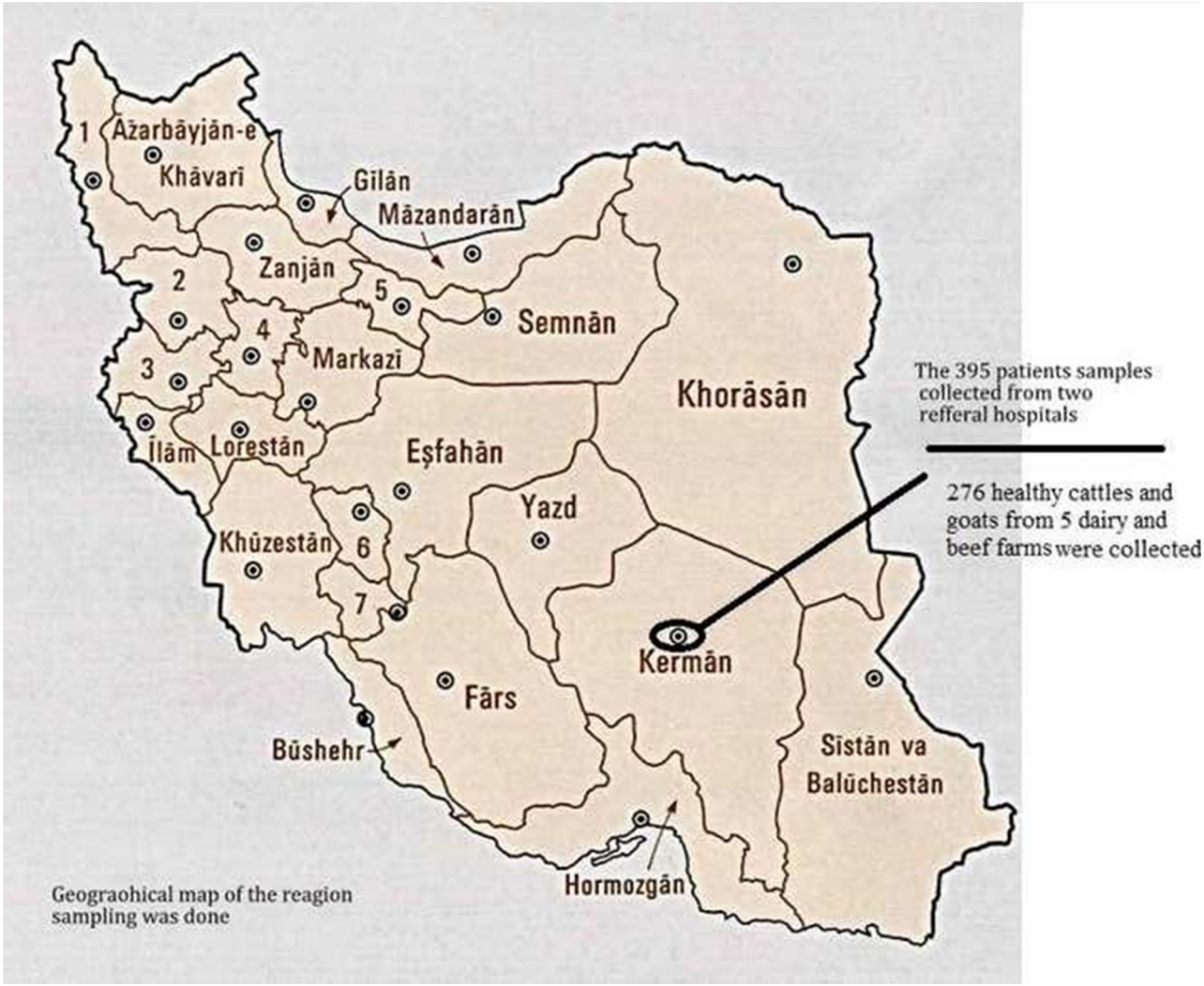



**Fig. 1.** The geographical map of samplings.

$bla_{SHV}$) as shown in Table 1. PCR was performed in a total volume of 25 µL, each containing of 12.5 µL ready to use master mix (Ampliqon Denmark), 1 µL of 10 pM each primer (Macrogen, Seoul, Korea), 9 µL sterilized D/W and 2.5 µL template DNA. DNA amplification was conducted under the following conditions: initial denaturation at 94 $^0$C for 5 min followed by 35 cycles of 30 s denaturation at 94 $^0$C for 30 s, annealing temperature as shown in Table 1 for 60 s, extension at 72 $^0$C for 60 s, and a final extension at 72 $^0$C for 7 min. Agarose gel electrophoresis was carried out for 1 h at 100 V/Cm with 0.5x Tris-borate-EDTA (TBE) buffer pH-8.0 and the gel was stained with green viewer dye. The amplified PCR products was then visualized in a Gel Doc system (Oxford, UK) and photographed.

### 2.5. Biofilm formation

Biofilm formation was quantified under static condition by microtiter assay as described previously [28]. Briefly, 1:100 diluted pathogenic *E. coli* samples were inoculated into a 96 well microtiter plate containing 100 µL fresh sterile Tryptic Soy Broth (TSB) medium supplemented with 0.2% glucose. Growth was monitored after 24 h incubation at 37 $^0$C. Unbound cells were removed from wells by inversion of the microtiter plate. The cells were removed by thorough washing with phosphate-buffered saline (pH-7.2) and the wells allowed drying. The attached cells were stained with 0.1% (wt/vol) crystal violet solution (Sigma grade) for 30 min. For detachment of the cells, we added 20% (vol/vol) dimethyl sulfoxide in ethanol to each well. Optical density (OD) was then measured at 490 nm. Simultaneously, *Pseudomonas aeruginosa* PAO1 was used as biofilm positive strain.

### 2.6. Determination of phylogenetic groups

The *E. coli* pathotypes assigned to one of the four main phylogenetic group of A, B1, B2 and D based on the presence or absence of the genes *chuA* and *yjaA* and the DNA fragment *tspE4C2* as described by Clermont et al., [29]. The primers targeted these three markers are shown in Table 1.

### 2.7. Detection of class 1 and 2 integrons

A duplex-PCR targeting class 1 and 2 integrons integrase ($int_1$ and $int_2$) genes were performed to investigate the prevalence of integrons in the EPEC, ETEC and EHEC collections [30]. Primers and annealing temperatures are listed in Table 1. The specificity of each primer sequence was confirmed by BLAST program available in the NCBI (http://www.ncbi.nlm.nih.gov/BLAST/). The reaction mixture was consisted of 3 µL 10X PCR- buffer, 5 µL of extracted DNA (20–50 ng), 0.6 µL DNA Taq polymerase, 0.5 µL dNTPs Mix (10 mM), 1.5 µL (10 pM/µL) of each primer (forward and reverse sequences), 0.8 µL 50 mM $MgCl_2$, and 13.6 µL sterile D/W. DNA amplification was conducted in temperature gradient thermal cycler (Biometra-T300 gradient, Gottingen, Germany)

**Table 1**
Genes, primers and sequences used in this study.

| Pathotype | Target gene | Primer sequence (5′–3′) | Size (bp) | Annealing temperature | Reference |
|---|---|---|---|---|---|
| EHEC | *stx* | F-GAGCGAAATAATTTATATGTG<br>R-TGATGATGGCAATTCAGTAT | 519 | 55$^0$C | [21] |
| | *eae* | F-CTGAACGGCGATTACGCGAA<br>R-CCAGACGATACGATCCAG | 917 | 55$^0$C | [22] |
| EPEC | *Bfp + eae* | F-AATGGTGCTTGCGCTTGCTGC<br>R-GCCGCTTTATCCAACCTGGTA | 326 | 55$^0$C | [22] |
| ETEC | *lt* | F-GGCGACAGATTATACCGTGC<br>R-CGGTCTCTATATTCCCTGTT | 450 | 56$^0$C | [22] |
| | *st* | F-TCTGTATTGTCTTTTTCACC<br>R-TTAATAGCACCCGGTACAAGC | 190 | 55$^0$C | [21] |
| ESBL genes | $bla_{CTX-M}$ | F- CGCTTTGCGATGTGCAG<br>R- ACCGCGATATCGTTGGT | 550 | 59 $^0$C | [27] |
| | $bla_{TEM}$ | F-GAGTATTCAACATTTCCGTGTC<br>R-AATCAGTGAGGCACCTATCTC | 561 | 59$^0$C | [26] |
| | $bla_{SHV}$ | F-TCAGCGAAAAACACCTTG<br>R-CCCGCAGATAAATCACCA | 471 | 56$^0$C | [26] |
| Integrons | *int1* | F-CAGTGGACATAAGCCTGTTC<br>R-CCCGAGGCATAGACTGTA | 160 | 55°C | [30] |
| | *int2* | F-TTGCGAGTATCCTAACCTG<br>R-TTACCTGCACTGGATTAAGC | 789 | 60°C | [30] |
| Genes for phylogenetic analysis | *ChuA* | F- GACGAACCAACGGTCAGGAT<br>R-TGCCGCCAGTACCAAAGACA | 279 | 59$^0$C | [29] |
| | *YjaA* | F- TGAAGTGTCAGGAGACGCTG<br>R-ATGGAGAATGCGTTCCTCAAC | 211 | 59$^0$C | [29] |
| | *TspE4.C2* | F-CTGGCG AAGACTGTATCAT<br>R-CGCGCCAACAAAGTATTA CG | 152 | 59$^0$C | [29] |
| MLVA markers | *ms06* | F-AAACGGGAGAGCCGGTTATT<br>R-TGTTGGTACAACGGCTCCTG | 39 | 55$^0$C | [31] |
| | *ms07* | F-GTCAGTTCGCCCAGACACAG<br>R-CGGTGTCAGCAAATCCAGAG | 39 | 55$^0$C | [31] |
| | *ms09* | F-GTGCCATCGGGCAAAATTAG<br>R-CCGATAAGGGAGCAGGCTAGT | 179 | 55 | [31] |
| | *ms11* | F-GAAACAGGCCCAGGCTACAC<br>R-CTGGCGCTGGTTATGGGTAT | 96 | 55$^0$C | [31] |
| | *ms21* | F-GCTGATGGCGAAGGAGAAGA<br>R-GGGAGTATGCGGTCAAAAGC | 141 | 55$^0$C | [31] |
| | *ms23* | F-GCTCCGCTGATTGACTCCTT<br>R-CGGTTGCTCGACCACTAACA | 375 | 55$^0$C | [31] |
| | *ms32* | F-TGAGATTGCCGAAGTGTTGC<br>R-AACTGGCGGCGTTTATCAAG | 101 | 55$^0$C | [31] |

with conditions as follows; initial DNA denaturation at 94 $^0$C for 10 min, followed by annealing temperatures (Table 1) for 40 s, with an extension at 72 $^O$C for 1 min and a final extension at 62 °C for 10 min. *Klebsiella pneumoniae* ATCC1029 containing class 1 and 2 integrons (kindly received from Institute Pasteur of Iran) was used as standard control strain.

### 2.8. Multiple locus variable number tandem repeat analysis

For MLVA analysis, genomics DNA of 57 *E. coli* pathotypes isolated in this study were extracted as described previously [31]. Briefly, a few colonies of *E. coli* isolates in Tryptic Soy agar (TSA) medium were added to sterile 200 µL D/W in DNase free Eppendorf vials, vortexes well and subjected to boiling temperature for 10 min. The cell debris were separated by centrifugation at 10.000 rpm for 7 min and the supernatants were transferred to new sterile vials for MLVA analysis. Here, the primers were constructed in order to target seven tandem repeat loci namely ms06, ms07, ms09, ms11, ms21, ms23, ms32 in all isolates as shown Table 1. The sequences were situated in five genes and two intergenic regions and had repeated motifs ranging from 6 to 30 base pairs. The PCR reactions for putative tandem repeat sequences from each genomic DNA [approx. 100 ng] were performed in 25 µL volume containing of 12.5 µL master mix (Ampliqon Denmark), 1 µL of 10 pM each primer (Macrogen, Seoul, Korea), 9 µL sterilized D/W and 2.5 µL template DNA. The temperature was raised to 94 $^0$C for 5 min, followed by thermal cycling consisted of 30 cycles, of 94 °C for 45 s, annealing temperature was set according to Table 1, extension at 72 °C for 60 s, with a final extension at 72 °C for 5 min. Each amplification reaction included a negative control (no-DNA template control). 3 µL amplified products were resolved by conventional electrophoresis through horizontal 1.5% (W/V) agarose gels using 25 bp size marker (Invitrogen) at 100 V/Cm for approximately 90 min.

Electrophoresis was conducted in 1 × TBE buffer (pH- 8.0) containing 0.5 µg/mL ethidium bromide (Sigma grade) and the results visualized and photographed in a Gel Doc system

(Oxford, England). Determination of the number of repeats in each locus was done using the Gene Marker software (Softgenetics, State College, Pennsylvania, USA). The dendrogram was generated and analyzed using the unweighted pair-group method with arithmetic averages (UPGMA) clustering employing BionNumerics version 7.6 (Applied Maths, Sint-MartensLatem, Belgium) software. The allele numbers were entered into BIONUMERICS as character values and a dendogram was constructed using categorical coefficients and the Ward algorithm. Isolates with 96% or greater similarity were considered as identical, and a cut-off value of 60% was selected for clustering. The repeat copy number calculated for each isolate using the following formula: number of repeat (base pair) = [size of each locus (bp) – flanking regions (bp)] /repeat size (bp) [32].

### 2.9. Statistical analyses

Statistical analysis was performed using SPSS 17.0. The statistical significance of the differences between the groups was tested by the $X^2$ test or Fisher's exact test. All p-values reported were two sided, and

**Table 2**
Antibiotic susceptibility EPEC, ETEC and EHEC pathotypes isolated from human and animal sources.

| ID | Pathotype | Source | Antibiotics | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | AUG | ATM | PRL | CIP | CRO | K | IMP | TN | AP | NA | AK | TS | CAZ | CXM |
| 7 | EPEC | Human | S | S | I | S | S | I | S | S | I | S | S | S | S | S |
| 10 | EPEC | Human | I | R | R | S | R | I | S | I | R | R | I | R | I | R |
| 19 | EPEC | Human | S | R | R | S | R | R | S | R | R | R | I | R | R | R |
| 32 | EPEC | Human | S | S | S | S | S | I | S | S | S | S | I | S | S | S |
| 40 | EPEC | Human | R | S | R | S | I | I | S | R | R | R | I | R | R | R |
| 66 | EPEC | Human | S | S | S | I | S | I | S | S | I | S | S | S | S | S |
| 75 | EPEC | Human | R | S | R | S | S | I | S | S | R | R | S | S | S | S |
| T75 | EPEC | Human | R | S | R | S | R | I | S | R | R | S | S | R | R | R |
| 77 | EPEC | Human | S | R | R | S | R | I | S | I | R | S | S | R | I | R |
| 79 | EPEC | Human | R | S | R | S | S | I | S | R | R | S | I | R | S | S |
| 92 | EPEC | Human | S | R | R | S | R | R | S | R | R | R | R | R | I | R |
| 93 | EPEC | Human | R | S | R | S | S | I | S | S | R | R | I | S | S | I |
| 107 | EPEC | Human | R | R | R | R | R | I | S | S | R | R | I | S | R | R |
| 144 | EPEC | Human | S | S | S | S | S | I | S | R | S | S | S | S | S | S |
| 172 | EPEC | Human | R | R | R | R | R | R | S | R | R | R | R | R | R | R |
| 176 | EPEC | Human | R | R | R | I | R | I | S | S | R | R | S | R | R | R |
| 181 | EPEC | Human | R | R | R | S | R | I | S | S | R | R | S | R | R | R |
| 183 | EPEC | Human | I | R | R | S | R | I | S | S | R | R | S | R | R | R |
| 187 | EPEC | Human | S | S | S | S | S | I | S | S | I | I | S | S | S | S |
| 296 | EPEC | Human | R | S | R | S | S | I | S | I | R | S | S | R | S | S |
| 309 | EPEC | Human | R | R | R | S | R | R | S | R | R | S | S | R | R | R |
| 333 | EPEC | Human | R | R | R | R | R | R | S | R | R | R | I | R | R | R |
| 336 | EPEC | Human | R | R | R | S | R | I | S | S | R | I | S | R | I | R |
| 357 | EPEC | Human | S | I | R | S | R | I | S | R | R | R | S | R | I | R |
| 370 | EPEC | Human | R | R | S | S | S | I | S | S | R | S | S | S | S | S |
| 385 | EPEC | Human | R | R | S | S | S | I | S | S | R | S | S | S | S | S |
| R27 | EPEC | Calve | S | S | S | S | I | R | S | R | I | R | R | R | I | I |
| DN115 | EPEC | Calve | S | R | S | I | I | S | S | S | S | R | S | R | S | I |
| 1150 | EPEC | Goat | S | S | S | S | S | S | S | R | I | S | I | S | S | I |
| 2003A | EPEC | Goat | S | R | S | I | I | S | S | S | S | I | S | S | R | I |
| 1084 | EPEC | Goat | S | S | S | S | S | S | S | R | S | S | S | S | S | I |
| R13 | EPEC | Calve | S | R | R | S | I | R | S | I | R | R | R | R | R | I |
| 1068 | EPEC | Goat | S | S | S | S | S | S | S | I | S | S | I | S | S | I |
| 2107A | EPEC | Goat | S | I | R | S | R | R | S | I | R | I | I | R | R | I |
| 2041A | EPEC | Goat | I | R | S | S | S | S | S | I | R | I | I | R | R | I |
| 2011 | EPEC | Goat | I | R | S | S | S | S | S | I | R | I | I | R | R | I |
| 2161B | EPEC | Goat | I | S | R | S | S | S | S | R | R | S | S | R | S | I |
| 2110A | EPEC | Goat | S | S | R | S | S | S | S | S | R | S | S | S | S | S |
| 235 | EPEC | Calve | S | S | R | S | S | S | S | S | R | S | R | S | S | S |
| 2089 | EPEC | Goat | S | S | S | S | S | S | S | I | S | S | S | S | S | I |
| 1028 | EHEC | Goat | S | S | S | S | S | S | S | R | S | S | S | S | S | S |
| 1059 | EHEC | Goat | S | S | S | S | S | R | S | R | S | S | I | S | S | S |
| 1095 | EHEC | Goat | S | S | S | S | S | R | S | S | S | R | S | S | S | S |
| 213 | EHEC | Calve | S | S | S | S | S | S | S | S | S | S | S | S | S | S |
| T32 | ETEC | Human | S | R | R | S | R | I | S | I | R | R | S | R | I | R |
| 168 | ETEC | Human | I | S | R | S | S | S | S | S | R | S | S | R | S | I |
| 192 | ETEC | Human | R | I | R | S | R | S | S | S | R | I | S | R | R | R |
| 199 | ETEC | Human | S | S | S | S | S | S | S | R | S | R | S | S | I | I |
| 201 | ETEC | Human | R | I | I | I | I | R | I | R | R | R | I | R | I | I |
| 249 | ETEC | Human | R | R | R | R | R | R | S | R | R | R | I | R | R | R |
| 294 | ETEC | Human | R | I | R | S | R | S | S | R | R | R | S | R | R | R |
| 295 | ETEC | Human | R | I | R | I | R | S | I | R | R | R | I | R | R | R |
| 349 | ETEC | Human | I | I | R | I | R | S | S | S | R | R | S | R | I | R |
| 360 | ETEC | Human | R | S | R | S | S | S | S | S | R | R | S | R | I | R |
| 391 | ETEC | Human | S | S | S | S | I | S | S | S | S | R | S | S | S | S |
| 429 | ETEC | Human | S | S | S | R | S | S | S | I | S | R | I | R | S | I |
| 444 | ETEC | Human | S | R | R | I | R | S | S | R | R | R | S | R | I | R |

**Abbreviations**: ATM = Aztreonam, AUG = amoxicillin/ clavulanic acid, PRL = Piperacillin, CIP = Ciprofloxacin, CRO = Ceftriaxone, K = Kanamycin, IMP = Imipenem, TN = Tobramycin, AP = Ampicillin, NA = Nalidixic acid, AK = Amikacin, TS = Trimethoprim-sulfamethoxazole, CAZ = Ceftazidime, CXM = Cefuroxime.

$p < 0.05$ was considered to be statistically significant.

## 3. Results

Out of 395 patients with diarrhea, 27% (n = 107) were aged ≤ 5 years old, 37% (n = 146) had 5–15 years old, 19% (n = 75) were between 40 years old and 17% (n = 67) had age ≥40 years old. Among the isolates, atypical EPEC was detected in 26 patients and 14 animal fecal samples, typical EPEC detected in 2 cattles, ETEC was found in stools of 13 patients with diarrhea and EHEC was recovered in 3 goats and one cattle, respectively.

We further studied antibiotic susceptibility of above isolates to different classes of antibiotics. The results are presented in Table 2. Fortunately, all the pathotypes were sensitive to imipenem, 75.4% were sensitive to ciprofloxacin but, 80.8% (n = 21) and 73.1% (n = 19) of the EPEC human isolates were resistant to ampicillin, trimethoprim-sulfamethoxazole (cotrimoxazole) and piperacillin, respectively. Similarly, we found 84.6% (n = 11) of the ETEC isolates were resistant to

**Table 3**
Antibiotic resistance patterns of *E. coli* pathotypes investigated in present study.

| Number of isolates | Number of antibiotics | Antibiotic resistance pattern |
|---|---|---|
| EPEC human isolates | | |
| 1 | 13 | ATM, PRL, AUG, CIP, CRO, K, TN,AP, TS,CAZ, CXM, NA, AK ** |
| 1 | 11 | ATM, PRL, AUG, CIP, CRO, K, TN, AP, TS, CAZ, CXM * |
| 1 | 10 | ATM, PRL, CRO,K,TN, AP, NA, TS, CXM, CAZ * |
| 1 | 10 | ATM, PRL, CRO, K, TN, AP, NA, TS, CXM, AK * |
| 1 | 10 | ATM, PRL, CRO, K,TN,AP,TS, CXM, CAZ, AUG |
| 1 | 9 | ATM, PRL, AUG, CRO, AP, NA, CAZ, CXM,CIP * |
| 2 | 9 | ATM, PRL, AUG, CRO, AP, NA, CAZ, CXM, TS |
| 1 | 8 | ATM, PRL, CRO, AP, NA, TS, CXM, CAZ |
| 1 | 7 | ATM, PRL, CRO, AP,NA,TS, CXM |
| 1 | 7 | PRL, TN, AP, NA,TS, CAZ, AUG |
| 1 | 7 | PRL, TN, AP,NA,TS, CXM, CRO |
| 1 | 7 | PRL,TN, AP, CAZ, AUG,CRO, TS |
| 1 | 6 | ATM, PRL, CRO, AP, CXM, AUG |
| 1 | 6 | ATM, PRL, CRO, AP, CXM, TS |
| 1 | 5 | PRL, AUG, AP, TS, TN |
| 1 | 4 | PRL, AUG, AP, TS |
| 2 | 4 | PRL, AUG, AP,NA(4) |
| 2 | 4 | ATM, AUG, AP, CXM |
| 1 | 1 | TN(1) |
| 4 | 0 | 0 |
| EPEC animal isolates | | |
| 1 | 8 | PRL, K, AP, NA, TS, CAZ, AK, ATM * |
| 1 | 6 | PRL, K, AP, CRO, TS, CAZ |
| 1 | 5 | K, TN, NA, AK, TS |
| 2 | 4 | ATM, AP,TS, CAZ |
| 1 | 4 | PRL, TN, AP, TS * |
| 1 | 3 | ATM, NA, TS |
| 1 | 3 | PRL, AP, AK |
| 2 | 1 | TN |
| 1 | 2 | PRL, AP |
| 1 | 2 | ATM, CAZ |
| 2 | 0 | 0 |
| ETEC human isolates | | |
| 1 | 12 | PRL, AUG, CRO, TN, AP,NA,TS, CAZ, CXM, ATM, CIP, K * |
| 2 | 9 | PRL, AUG, CRO,TN, AP, NA,TS, CAZ, CXM * |
| 1 | 8 | ATM, PRL, CRO, TN, AP, NA, TS, CXM |
| 1 | 7 | ATM,PRL, CRO, AP, NA, TS, CXM |
| 1 | 7 | PRL, AUG, CRO, AP, TS, CAZ, CXM |
| 1 | 6 | PRL, CRO, AP, NA, TS, CXM |
| 1 | 6 | PRL, AUG, AP, NA, TS, CXM |
| 1 | 6 | AUG, K, TN, AP, NA, TS |
| 1 | 3 | CIP, NA, TS |
| 1 | 3 | PRL, AP, TS |
| 1 | 2 | TN, NA |
| 1 | 1 | TN |
| EHEC animal isolates | | |
| 1 | 1 | K, TN |
| 1 | 1 | K, NA |
| 1 | 1 | TN |
| 1 | 0 | 0 |

0 = Not resistance to antibiotics.
* Multi-drug resistance.
** Extensive drug resistance.

nalidixic acid and co-trimoxazole but sensitive to amikacin (Table 2). In contrast, EHEC isolates were susceptible to the most of antibiotics tested ($P \leq 0.05$). The distribution of antimicrobial resistance profiles within the EPEC, ETEC and EHEC pathotypes are presented in Table 3. As the results indicate, of 26 EPEC human isolates, 50% (n = 13) were multi-drug resistance (MDR) exhibiting resistance to at least three classes of antibiotics.

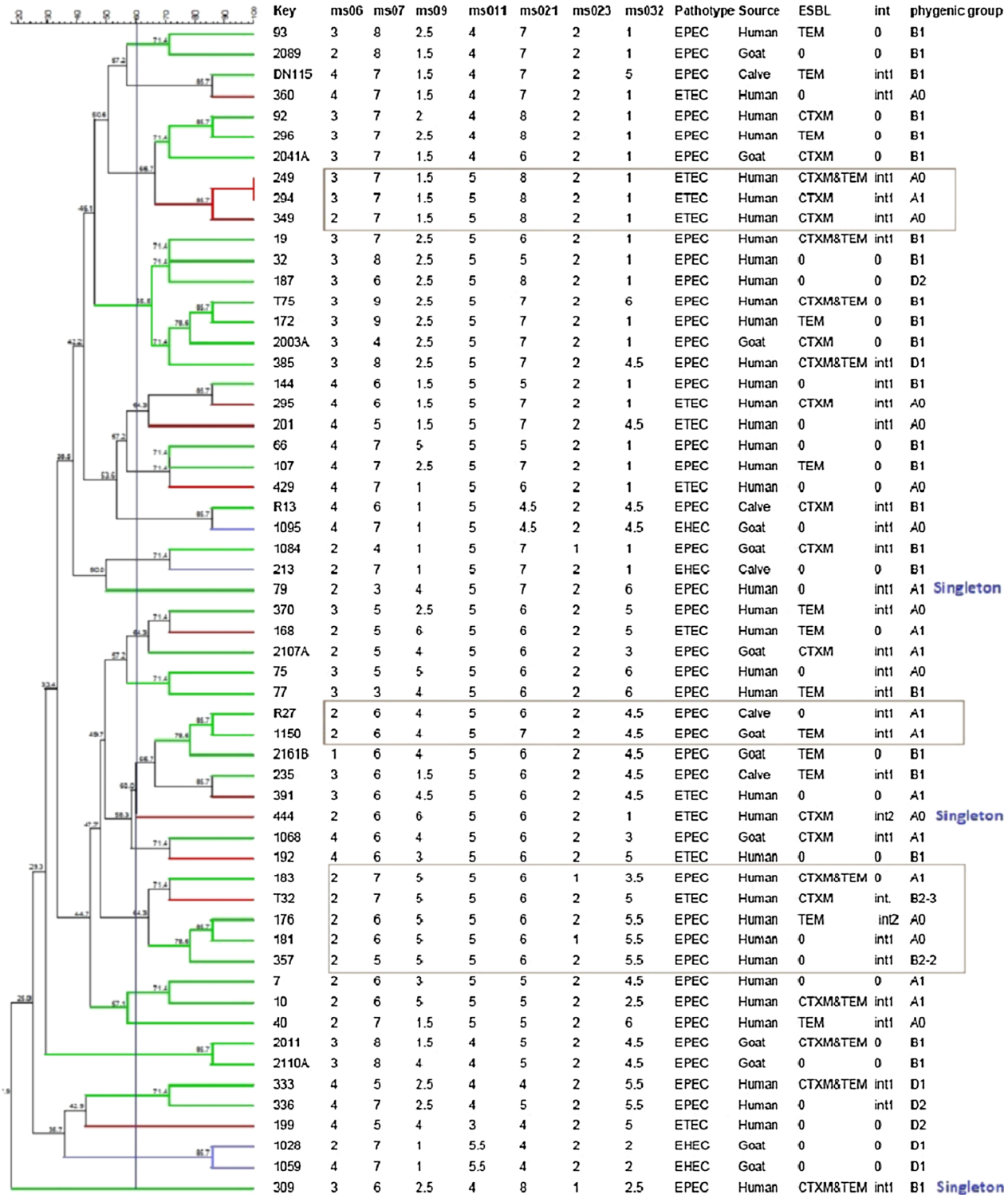
Interestingly, one EPEC isolate showed extensive drug resistance (XDR). Comparing to human EPEC isolates, only 36% (n = 5) EPEC animal isolates were resistant to three classes of antibiotics (Table 3). In case of ETEC, among 13 isolates, 61.5% (n = 8) showed different MDR profiles. There was a significant difference between the antibiotic resistance patterns of the EPEC, ETEC and EHEC pathotypes ($p \leq 0.05$). We further investigated the prevalence of ESBL genes in the above *E. coli* isolates. The most commonly observed genes were $bla_{TEM}$ and $bla_{CTX\text{-}M}$, detected in tes, while isolates harboured both $bla_{CTX\text{-}M}$ and $bla_{TEM,}$ genes simultaneously. The $bla_{SHV}$ gene was absent in this study. Interestingly, those isolates carried these genes showed MDR property (Fig. 2). Furthermore, 57.5% (n = 23) of EPEC and 46.15% (n = 6) of ETEC isolates carried class 1 integrons, while class 2 integrons was detected in one EPEC (strain 176) and one ETEC (strain 444), respectively (Fig. 2). None of the isolates had both integrons together. There was a significant relationship between antibiotic resistance, $bla_{CTX\text{-}M}$ gene and presence of class 1 integrons among the EPEC pathotype ($p \leq 0.05$). Further study on the biofilm formation revealed that, 3 out of 4 EHEC isolates showed strong biofilm, while majority our EPEC isolates were capable of producing moderate or no biofilm and ETEC isolates were mainly produced moderate and weak biofilm (Fig. 3).

The phylogenetic groups of the above pathotypes were further studied. We found 34.6% (n = 9) of EPEC human isolates were belonged to phylogroup A (A0, A1), 50% (n = 13) were belonged to phylogroup B (B1) and 15.3% (n = 4) were belonged to phylogroup D (D1, D2) as shown in Fig. 2. Similarly, we found that, 28.5% (n = 4) animal EPEC were identified as group A, 71.5% (n = 10) as group B (B1). In case of ETEC human isolates, 77% (n = 10) were attributed to phylogenetic group A, 15.3% (n = 2) were phylogroup B (B1,B2) and 7.7% (n = 1) were classified as phylogroup D. For EHEC isolates, 25% (n = 2) were belonged to phylogroups A and B, while 50% (n = 2) were identified as phylogroup D by PCR method (Fig. 2).

The MLVA results exhibited high polymorphism in seven VNTR loci. Dendogram analysis revealed three main clusters which classified into 2 clonal complexes and three singletons (Fig. 2). The cluster 1 were consist of three ETEC isolates differed by one VNTR, all carried class 1 integrons, harbored either CTX-M or TEM-1 β-lactamases and belonged to phylogroup A. Cluster 2 consist of 5 EPEC human isolates differed by one or two VNTER marker, and belongs to phylogroup B and A. Cluster 3 was consist of two EPEC animal isolates both harbored class 1 integrons and $bla_{TEM}$ gene and belonged to phylogroup A (Fig. 2). Interestingly, isolates with known epidemiological sources (two ETEC isolates; ID 249 and ID 294) showed identical MLVA pattern. The remaining isolates exhibited scattered fingerprints differed in either two or more VNTR allelic variants. Furthermore, the EHEC isolates from goats showed identical VNTR behavior, while the isolate obtained from cattles exhibited completely different MLVA pattern (Fig. 2). There was no any epidemiological relationship in MLVA patterns.

## 4. Discussion

According to the World Health Organization (WHO) report, approximately 11 million children under the age of 5 years die due to *E. coli* mediated gastroenteritis. Among DECs, enteropathogenic atypical EPEC was a primary etiological agent of diarrhea in children and adults accounts for 5% to 10% of pediatric diarrhea in resource poor countries [33]. Pathogenic *E. coli* are endemic in south and southeast of Iran and



| Key | ms06 | ms07 | ms09 | ms011 | ms021 | ms023 | ms032 | Pathotype | Source | ESBL | int | phygenic group | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 93 | 3 | 8 | 2.5 | 4 | 7 | 2 | 1 | EPEC | Human | TEM | 0 | B1 | |
| 2089 | 2 | 8 | 1.5 | 4 | 7 | 2 | 1 | EPEC | Goat | 0 | 0 | B1 | |
| DN115 | 4 | 7 | 1.5 | 4 | 7 | 2 | 5 | EPEC | Calve | TEM | int1 | B1 | |
| 360 | 4 | 7 | 1.5 | 4 | 7 | 2 | 1 | ETEC | Human | 0 | int1 | A0 | |
| 92 | 3 | 7 | 2 | 4 | 8 | 2 | 1 | EPEC | Human | CTXM | 0 | B1 | |
| 296 | 3 | 7 | 2.5 | 4 | 8 | 2 | 1 | EPEC | Human | TEM | 0 | B1 | |
| 2041A | 3 | 7 | 1.5 | 4 | 6 | 2 | 1 | EPEC | Goat | CTXM | 0 | B1 | |
| 249 | 3 | 7 | 1.5 | 5 | 8 | 2 | 1 | ETEC | Human | CTXM&TEM | int1 | A0 | |
| 294 | 3 | 7 | 1.5 | 5 | 8 | 2 | 1 | ETEC | Human | CTXM | int1 | A1 | |
| 349 | 2 | 7 | 1.5 | 5 | 8 | 2 | 1 | ETEC | Human | CTXM | int1 | A0 | |
| 19 | 3 | 7 | 2.5 | 5 | 6 | 2 | 1 | EPEC | Human | CTXM&TEM | int1 | B1 | |
| 32 | 3 | 8 | 2.5 | 5 | 5 | 2 | 1 | EPEC | Human | 0 | 0 | B1 | |
| 187 | 3 | 6 | 2.5 | 5 | 8 | 2 | 1 | EPEC | Human | 0 | 0 | D2 | |
| T75 | 3 | 9 | 2.5 | 5 | 7 | 2 | 6 | EPEC | Human | CTXM&TEM | 0 | B1 | |
| 172 | 3 | 9 | 2.5 | 5 | 7 | 2 | 1 | EPEC | Human | TEM | 0 | B1 | |
| 2003A | 3 | 4 | 2.5 | 5 | 7 | 2 | 1 | EPEC | Goat | CTXM | 0 | B1 | |
| 385 | 3 | 8 | 2.5 | 5 | 7 | 2 | 4.5 | EPEC | Human | CTXM&TEM | int1 | D1 | |
| 144 | 4 | 6 | 1.5 | 5 | 5 | 2 | 1 | EPEC | Human | 0 | int1 | B1 | |
| 295 | 4 | 6 | 1.5 | 5 | 7 | 2 | 1 | ETEC | Human | CTXM | int1 | A0 | |
| 201 | 4 | 5 | 1.5 | 5 | 7 | 2 | 4.5 | ETEC | Human | 0 | int1 | A0 | |
| 66 | 4 | 7 | 5 | 5 | 5 | 2 | 1 | EPEC | Human | 0 | 0 | B1 | |
| 107 | 4 | 7 | 2.5 | 5 | 7 | 2 | 1 | EPEC | Human | TEM | 0 | B1 | |
| 429 | 4 | 7 | 1 | 5 | 6 | 2 | 1 | ETEC | Human | 0 | 0 | A0 | |
| R13 | 4 | 6 | 1 | 5 | 4.5 | 2 | 4.5 | EPEC | Calve | CTXM | int1 | B1 | |
| 1095 | 4 | 7 | 1 | 5 | 4.5 | 2 | 4.5 | EHEC | Goat | 0 | int1 | A0 | |
| 1084 | 2 | 4 | 1 | 5 | 7 | 1 | 1 | EPEC | Goat | CTXM | int1 | B1 | |
| 213 | 2 | 7 | 1 | 5 | 7 | 2 | 1 | EHEC | Calve | 0 | 0 | B1 | |
| 79 | 2 | 3 | 4 | 5 | 7 | 2 | 6 | EPEC | Human | 0 | int1 | A1 | Singleton |
| 370 | 3 | 5 | 2.5 | 5 | 6 | 2 | 5 | EPEC | Human | TEM | int1 | A0 | |
| 168 | 2 | 5 | 6 | 5 | 6 | 2 | 5 | ETEC | Human | TEM | 0 | A1 | |
| 2107A | 2 | 5 | 4 | 5 | 6 | 2 | 3 | EPEC | Goat | CTXM | int1 | A1 | |
| 75 | 3 | 5 | 5 | 5 | 6 | 2 | 6 | EPEC | Human | 0 | int1 | A0 | |
| 77 | 3 | 3 | 4 | 5 | 6 | 2 | 6 | EPEC | Human | TEM | int1 | B1 | |
| R27 | 2 | 6 | 4 | 5 | 6 | 2 | 4.5 | EPEC | Calve | 0 | int1 | A1 | |
| 1150 | 2 | 6 | 4 | 5 | 7 | 2 | 4.5 | EPEC | Goat | TEM | int1 | A1 | |
| 2161B | 1 | 6 | 4 | 5 | 6 | 2 | 4.5 | EPEC | Goat | TEM | 0 | B1 | |
| 235 | 3 | 6 | 1.5 | 5 | 6 | 2 | 4.5 | EPEC | Calve | TEM | int1 | B1 | |
| 391 | 3 | 6 | 4.5 | 5 | 6 | 2 | 4.5 | ETEC | Human | 0 | 0 | A1 | |
| 444 | 2 | 6 | 6 | 5 | 6 | 2 | 1 | ETEC | Human | CTXM | int2 | A0 | Singleton |
| 1068 | 4 | 6 | 4 | 5 | 6 | 2 | 3 | EPEC | Goat | CTXM | int1 | A1 | |
| 192 | 4 | 6 | 3 | 5 | 6 | 2 | 5 | ETEC | Human | 0 | 0 | B1 | |
| 183 | 2 | 7 | 5 | 5 | 6 | 1 | 3.5 | EPEC | Human | CTXM&TEM | 0 | A1 | |
| T32 | 2 | 7 | 5 | 5 | 6 | 2 | 5 | ETEC | Human | CTXM | int. | B2-3 | |
| 176 | 2 | 6 | 5 | 5 | 6 | 2 | 5.5 | EPEC | Human | TEM | int2 | A0 | |
| 181 | 2 | 6 | 5 | 5 | 6 | 1 | 5.5 | EPEC | Human | 0 | int1 | A0 | |
| 357 | 2 | 5 | 5 | 5 | 6 | 2 | 5.5 | EPEC | Human | 0 | int1 | B2-2 | |
| 7 | 2 | 6 | 3 | 5 | 5 | 2 | 4.5 | EPEC | Human | 0 | 0 | A1 | |
| 10 | 2 | 6 | 5 | 5 | 5 | 2 | 2.5 | EPEC | Human | CTXM&TEM | int1 | A1 | |
| 40 | 2 | 7 | 1.5 | 5 | 5 | 2 | 6 | EPEC | Human | TEM | int1 | A0 | |
| 2011 | 3 | 8 | 1.5 | 4 | 5 | 2 | 4.5 | EPEC | Goat | CTXM&TEM | 0 | B1 | |
| 2110A | 3 | 8 | 4 | 4 | 5 | 2 | 4.5 | EPEC | Goat | 0 | 0 | B1 | |
| 333 | 4 | 5 | 2.5 | 4 | 4 | 2 | 5.5 | EPEC | Human | CTXM&TEM | int1 | D1 | |
| 336 | 4 | 7 | 2.5 | 4 | 5 | 2 | 5.5 | EPEC | Human | 0 | int1 | D2 | |
| 199 | 4 | 5 | 4 | 3 | 4 | 2 | 5 | ETEC | Human | 0 | 0 | D2 | |
| 1028 | 2 | 7 | 1 | 5.5 | 4 | 2 | 2 | EHEC | Goat | 0 | 0 | D1 | |
| 1059 | 4 | 7 | 1 | 5.5 | 4 | 2 | 2 | EHEC | Goat | 0 | 0 | D1 | |
| 309 | 3 | 6 | 2.5 | 4 | 8 | 1 | 2.5 | EPEC | Human | CTXM&TEM | int1 | B1 | Singleton |

**Fig. 2.** MLVA profiles of EPEC, ETEC and EHEC isolates recovered in patients with diarrhea and animal sources. The MLVA was repeated twice.

majority of children suffered from chronic type of diarrhea such as abdominal pain, constipation and loss of weight. There are a few investigations on prevalence and epidemiology of the DEC pathotypes in this region [17,18] therefore, prevalence of different pathotypes of DEC will provide the critical information for determining appropriate therapies for patients with suspected *E. coli* infections and controlling of the outbreak. Considering cattles and goats as main source of EPEC and EHEC transmission to human in this region, attempt was made to address the prevalence, antibiotic resistant profile and phylogroups studies and possible genetic linkage by MLVA typing. In this investigation, we found that, the frequency of EPEC, ETEC and EHEC among human and animal populations were low (57/671 isolates). Our results were in accordance with study from India which suggest that, EPEC pathotype seen in 39 (9.9%) of cases diarrhea in children under 5 years old followed by ETEC in 16 (4.1%) cases [34].

Furthermore, in our study, atypical EPEC was more commonly seen cases while typical EPEC was detected in animal stool samples. Majority of EPEC and ETEC human isolates showed MDR profiles. Ampicillin,

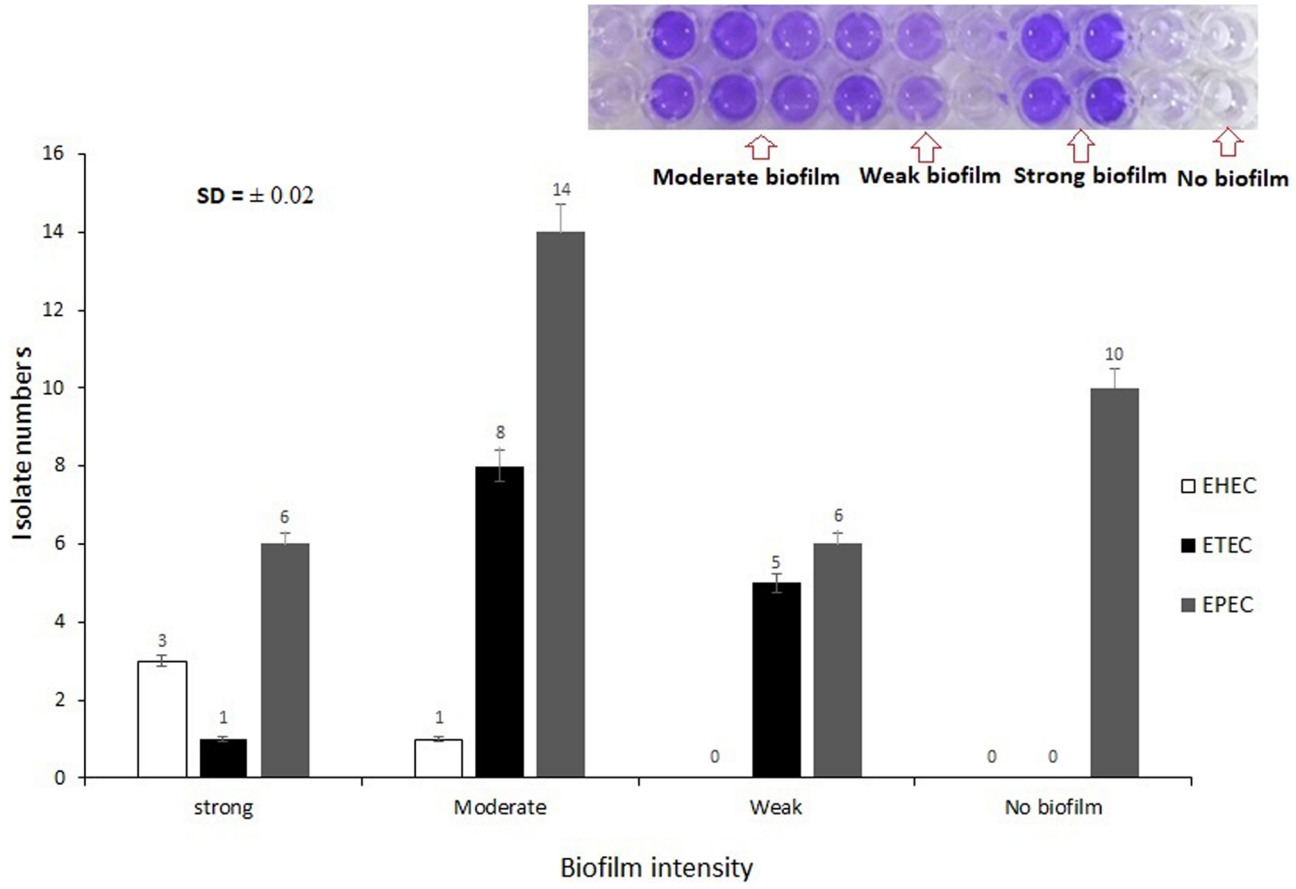



**Fig. 3.** Biofilm formation among EPEC, ETEC and EHEC isolates. The above results are average of three replicates with standard deviation 0.02. The biofilm was stained with crystal violet dye and intensity of the biofilm was measured at optical density 480 nm.

trimethoprim-sulphamethoxazole and fluoroquinolones are widely used as the first choice drug for treatment of intestinal tract infections because of their ready availability and low cost. In the current study, resistance to commonly used antibiotics such as ampicillin and co-trimoxazole, was in accordance with previous findings from other countries, such as; Vietnam, Mexico, Argentina, Tanzania, and Peru [31]. Since these antibiotics are widely used to treat diarrhea, it is therefore not surprising that we found higher resistance rates against these antimicrobial agents among our isolates. Although imipenem is not indicated to treat diarrhea, we tested the susceptibilities of the isolates to this antibiotic, since it could be empirically used for treatment of diarrheal disease. Fortunately, none of the isolates were resistant to this antimicrobial agent, indicating that imipenem is the most effective agents against our isolates. MDR strains of diarrheagenic *E. coli* pose a serious clinical challenge. The MDR phenotype can emerge due to the presence of ESBLs enzymes. In our study, the majority of

In addition, EPEC and ETEC isolates encoded either $bla_{\text{CTX-M}}$ or $bla_{\text{TEM-1}}$ genes but not $bla_{\text{SHV}}$. It is very important to determine the prevalence of ESBLs within a geographic area as emergence of ESBL-producing *E coli* leads to higher reliance for treatment with carbapenems. Bok et al., reported in the bovine isolates, the percentage of resistant *E. coli* derived from dairy cows was higher than from beef cows [35].

We also studied relationship between biofilm formation and antibiotic resistances profile of above isolates. The highest prevalence of antibiotic resistance was observed in moderate and weak biofilm producers. The results showed that, there was no statistically significant relationship between antibiotic resistance profiles and ability to form strong biofilm in EPEC and ETEC isolates. Similar observations were made previously by Pavlickova et al., [36].

We observed a similar phylogenetic conformation in the *E. coli* populations from healthy cows and goats which were mainly classified as A; but also B1 and D were detected; human isolates were assigned to group B2 and D2. The results of MLVA were interesting because no any clonal relation between human and animal isolates were observed (the isolates demonstrated scattered fingerprints). Therefore, we suggest that diarrheagenic *E. coli* investigated in this region were not epidemiologically linked together. This finding is in agreement with previous reports that documented a particularly high polymorphism rate for EPEC and ETEC pathotypes [37]. Staples et al. [38], studied 61 EPEC strains isolated from stool specimens of symptomatic persons from 2008 to 2011 for the prevalence of diarrhea-associated putative genes. Phylogenetic typing, serotyping and MLVA showed the isolates were highly heterogeneous, representing all 4 phylogenetic groups and comprising 59 MLVA types.

## 5. Conclusion

In conclusion, MLVA data showed high polymorphisms among EPEC, ETEC and EHEC strains isolated from a variety of sources.

However, presence of EHEC in animal fecal samples poses public health concern as transmission through food chain must be considered. Antimicrobial agents, often used in livestock, belong to the same classes than those administered in human treatments. Thus, the application of antibiotics in farms animals produces a selective pressure allowing the dissemination of resistant bacteria, which represents a risk to human health. Further epidemiological studies are required to explore these sources in context with occurrence of human cases.

## Conflict of interest

The authors declare that they have no conflict of interest.

## Funding

This study supported in part by a grant from the Research Grants Committee Kerman University of Medical Sciences, Iran (grant number 92/403) awarded to Dr. Shakibaie MR.

## Acknowledgments

The authors would like to thank the staff members of the Research Center for Infectious Diseases and Tropical Medicine, Department of Microbiology and Virology, Kerman University of Medical Sciences (Kerman, Iran) for their help during this investigation.

## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.cimid.2019.01.004.

## References

[1] J.P. Nataro, J.B. Kaper, Diarrheagenic *Escherichia coli*, Clin. Microbiol. Rev. 11 (1) (1998) 142–201.
[2] B. Hebbelstrup, K.E.P. Olsen, C. Struve, K.A. Krogfelt, A.M. Petersen, Epidemiology and clinical manifestations of enteroaggregative Escherichia coli, Clin. Microbiol. Rev. 27 (3) (2014) 614–630.
[3] H.D. Chen, G. Frankel, Enteropathogenic *Escherichia coli*: unravelling pathogenesis, FEMS Microbiol. Rev. 29 (1) (2005) 83–98.
[4] V. Shetty, A. Shetty, I. Karunasagar, S. Kumar, Prevalence and characterization of diarrheagenic *Escherichia coli* isolated from adults and children in Mangalore India, J. Lab. Phys. 4 (1) (2012) 24–29.
[5] A.E. Jerse, J. Yu, B.D. Tall, J.B. Kaper, A genetic locus of enteropathogenic *Escherichia coli* necessary for the production of attaching and effacing lesions on tissue culture cells, Proc. Natl. Acad. Sci. U. S. A. 87 (20) (1990) 7839–7843.
[6] A.C. Moreno, A.F. Filho, T.D. Gomez, S.T. Ramos, L.P. Montemor, V.C. Travares, S. Filho-Ldos, K. Irino, M.B. Martinez, Etiology of childhood diarrhea in the northeast of Brazil: significant emergent diarrheal pathogeneses, Diagn. Microbiol. Infect. Dis. 66 (1) (2010) 50–57.
[7] I. Aktan, K.A. Sprigings, R.M. La Ragione, L.M. Faulkner, G.A. Paiba, M.J. Woodward, Characterization of attaching-affacing *Escherichia coli* isolated from animals at slaughter in England and Wales, Vet. Microbiol. 102 (1-2) (2004) 43–53.
[8] D. Müller, L. Greune, G. Heusipp, H. Karch, A. Fruth, H. Tschape, A. Schmidt, Identification of unconventional intestinal pathogenic *Escherichia coli* isolates expressing intermediate virulence factor profiles by using a novel single-step multiplex PCR, Appl. Environ. Microbiol. 73 (10) (2007) 3380–3390.
[9] A. von Mentzer, T.R. Connor, L.H. Wieler, T. Semmler, A. Lguchi, N.R. Thomson, D.R. Rasko, E. Joffre, J. Corander, D. Pickard, G. Wiklund, A.M. Svennerholm, A. Sjöling, G. Dougan, Identification of enterotoxigenic *Escherichia coli* (ETEC) clades with long-term global distribution, Nat. Genet. 46 (12) (2014) 1321–1326.
[10] Y. Nguyen, V. Sperandio, Enterohemorrhagic *E. coli* (EHEC) pathogenesis, Front. Cell. Infect. Microbiol. 2 (2012) 1–7, https://doi.org/10.3389/fcimb.2012.00090.
[11] J. Thévenot, L. Etienne-Mesmin, S. Denis, S. Chalancon, M. Alric, V. Livrelli, S. Blanquet-Diot, Enterohemorrhagic *Escherichia coli* O157: H7 survival in an in vitro model of the human large intestine and interactions with probiotic yeasts and resident microbiota, Appl. Environ. Microbiol. 79 (3) (2013) 1058–1064.
[12] L. Hall-Stoodley, J.W. Costerton, P. Stoodley, Bacterial biofilms: from the natural environment to infectious diseases, Nat. Rev. Microbiol. 2 (2) (2004) 95–108.
[13] J.W. Costerton, Z. Lewandowski, D.E. Caldwell, D.R. Korber, H.M. Lappin-Scott, Microbial biofilms, Annu. Rev. Microbiol. 49 (1995) 711–745.
[14] M.A. Schembri, K. Kjaergaard, P. Klemm, Global gene expression in *Escherichia coli* biofilms, Mol. Microbiol. 48 (1) (2003) 253–267.
[15] R. Simm, M. Morr, A. Kader, M. Nimtz, U. Römling, GGDEF and EAL domains inversely regulate cyclic di-GMP levels and transition from sessility to motility, Mol. Microbiol. 53 (4) (2004) 1123–1134.
[16] I. Løbersli, A.L. Wester, A. Kristiansen, L.T. Brandal, Molecular differentiation of Shigella Spp. from Enteroinvasive*E. coli*, Eur. J. Microbiol. Immunol. 6 (3) (2016) 197–205.
[17] R. Taghadosi, M.R. Shakibaie, H. Alizade, H. Hosseini-Nave, A. Askari, R. Ghanbarpour, Serogroups, subtypes and virulence factors of shiga toxin-producing Escherichia coli isolated from human, calves and goats in Kerman, Iran, Gastroenterol. Hepatol. Bed. Bench. 11 (1) (2018) 60–67.
[18] H. Hosseini-Nave, M.S. Salavati, M. Lashkari, M.A. Ravari, Z. Hashemizadeh, F.S. Morghaki, Distribution of genes encoding virulence factors and multilocus variablenumber tandem-repeat analysis (MLVA) of entero-aggregative *Escherichia coli* (EAEC) isolated in Iran from patients with diarrhoea, J. Med. Microbiol. 67 (9) (2018) 1334–1339.
[19] A.E. Heuvelink, N.C. Van De Kar, J.F. Meis, L.A. Monnens, W.J. Melchers, Characterization of verocytotoxin-producing *Escherichia coli* O157 isolates from patients with haemolytic uremic syndrome in Western Europe, Epidemiol. Infect. 115 (1) (1995) 1–14.
[20] H. Hosseini Nave, S. Mansouri, M. Emaneini, M. Moradi, Distribution of genes encoding virulence factors and molecular analysis of Shigella spp. isolated from patients with diarrhea in Kerman, Iran, Microb. Pathog. 92 (2016) 68–71, https://doi.org/10.1016/j.micpath.2015.11.015.
[21] H. Kobayashi, J. Shimada, M. Nakazawa, T. Morozumi, T. Pohjanvirta, S. Pelkonen, Prevalence and characteristics of shiga toxin-producing *Escherichia coli* from healthy cattle in Japan, Appl. Environ. Microbiol. 67 (1) (2001) 484–489.
[22] K.R.S. Aranda, S.H. Fabbricotti, U. Fagundes-Neto, I.C.A. Scaletsky, Single multiplex assay to identify simultaneously enteropathogenic, enteroaggregative, enterotoxigenic, enteroinvasive and Shiga toxin-producing *Escherichia coli* strains in Brazilian children, FEMS Microbiol. Lett. 267 (2) (2007) 145–150.
[23] H. Salmani, A. Azarnezhad, M.R. Fayazi, A. Hosseini, Pathotypic and phylogenetic study of diarrheagenic *Escherichia coli* and uropathogenic *E. coli*using Multiplex-Polymerase Chain Reaction, Jundishapur, J. Microbiol. 9 (2) (2016) e28331, , https://doi.org/10.5812/jjm.28331.
[24] CLSI, Performance Standards for Antimicrobial Susceptibility Testing: Twenty-second Informational Supplement, CLSI Document M100-S22, Clinical and Laboratory Standard Institute, Wayne, PA, 2016.
[25] H. Ejaz, I. ul-Haq, S. Mahmood, A. Zafar, M.M. Javed, Detection of extended-spectrum β-lactamases in *Klebsiella pneumoniae*: comparison of phenotypic characterization methods, Pak. J. Med. Sci. 29 (3) (2013) 768–772.
[26] F.R. Zaniani, Z. Meshkat, M. Naderinasab, M. Khaje-Karamadini, K. Ghazvini, A. Rezaee, H. Esmaily, M. Nabavinia, M.D. Hoseini, The prevalence of TEM and SHV genes among Extended-Spectrum β-Lactamases producing *Escherichia coli* and *Klebsiella Pneumoniae*, Iran. J. Basic Med. Sci. 15 (1) (2012) 654–660.
[27] C. Dutour, R. Bonnet, H. Marchandin, M. Boyer, C. Chanal, D. Sirot, J. Sirot, CTXM-1, CTX-M-3, and CTX-M-14 β-Lactamases from Enterobacteriaceae isolated in France, Antimicrob. Agents Chemother. 46 (2) (2002) 534–537.
[28] G.A.O. Toole, Microtiter dish biofilm formation assay, J. Vis. Exp. 30 (47) (2011) 10–11.
[29] O. Clermont, S. Bonacorsi, E. Bingen, Rapid and simple determination of the Escherichia coli phylogenetic group, Appl. Environ. Microbiol. 66 (10) (2000) 4555–4558.
[30] Z.Q. Yan, D.X. Shen, J.R. Cao, R. Chen, X. Wei, L.P. Liu, et al., Susceptibility patterns and molecular epidemiology of multidrug-resistant *Acinetobacter baumannii* strains from three military hospitals in China, Int. J. Antimicrob. Agents 35 (3) (2010) 269–273.
[31] O. Gorgé, S. Lopez, V. Hilaire, O. Lisanti, V. Ramisse, G. Vergnaud, Selection and validation of a multi-locus variable-number tandem-repeat analysis panel for typing Shigella spp, Clin. Microbiol. 46 (3) (2008) 1026–1036.
[32] M. Memariani, S. Najar Peerayeh, T. Zahraei Salehi, S.K. Mostafavi, Occurrence of SHV, TEM and CTX-M β-Lactamase genes among enteropathogenic *Escherichia coli* strains isolated from children with diarrhea, Jundishapur, J. Microbiol. 8 (4) (2015) e15620, , https://doi.org/10.5812/jjm.8(4)015.15620.
[33] WHO, Diarrhoeal Disease, (2013) http://www.who.int/mediacentre/factsheets/fs330/en/index.html.
[34] P. Rajendrana, S. Ajjampur, D. Chidambaram, G. Chandrabose, B. Thangaraj, R. Sarkar, P. Samuel, G. Kang, Pathotypes of diarrheagenic *Escherichia coli* in children attending a tertiary care hospital in South India, Diagn. Microbiol. Infect. Dis. 68 (2) (2010) 117–122.
[35] E. Bok, J. Mazurek, M. Stosik, M. Wojciech, K. Baldy-Chudzik, Prevalence of virulence determinants and antimicrobial resistance among commensal Escherichia coli derived from dairy and beef cattle, Int. J. Environ. Res. Public Health 12 (1) (2015)

970–985, https://doi.org/10.3390/ijerph120100970.
[36] S. Pavlickova, A. Klancnik, M. Dolezalova, S.S. Mozina, I. Holko, Antibiotic resistance, virulence factors and biofilm formation ability in *Escherichia coli* strains isolated from chicken meat and wildlife in the Czech Republic, J. Environ. Sci. Health B 52 (8) (2017) 570 -576.
[37] T.A.T. Gomes, W.P. Elias, I.C.A. Scaletsky, B.E.C. Guth, J.F. Rodrigues, R.M.F. Piazza, Diarrheagenic Escherichia coli, Braz. J. Microbiol. 47 (1) (2016) 3–30, https://doi.org/10.1016/j.bjm.2016.10.015.
[38] M. Staples, C.J. Doyle, R.A.M. Graham, A.V. Jennison, Molecular epidemiological typing of enteropathogenic *Escherichia coli* strains from Australian patients, Diagn. Microbiol. Infect. Dis. 75 (3) (2013) 320–324, https://doi.org/10.1016/j.diagmicrobio.2012.11.010.