

Distribution of Shiga toxin genes *subtypes* in B₁ phylotypes of *Escherichia coli* isolated from calves suffering from diarrhea in Tehran suburb using DNA oligonucleotide arrays

Hamid Staji^{1*}, Alfreda Tonelli², Abbas Javaheri-Vayeghan¹, Emad Changizi¹, Mohammad Reza Salimi-Bejestani¹

¹Department of Pathobiology, Faculty of Veterinary Medicine, Semnan University, Semnan, Iran.

²Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise "G. Caporale," Research and Development, Campo Boario, 64100 Teramo, Italy.

Received: January 2015, Accepted: June 2015

ABSTRACT

Background and Objectives: Shiga toxin-producing *Escherichia coli* (STEC) have emerged as human pathogens and contamination via animal origin has been a major public health concern. We compared the distribution of phylogenetic groups and prevalence of *stx* gene variants among the pathogenic strains of *Escherichia coli* isolated from feces of diarrheatic calves in Tehran suburb farms.

Materials and Methods: In this study we screened 140 diarrheatic calves (1-15 days old) for *E. coli* strains during a 3 months period of time. The isolated strains were grouped into different phylotypes according to the presence of *chuA*, *yjaA* and *TSPE4.C2* genes. Then, the prevalence of *stx* gene subtypes was evaluated in the B₁ phylotypes.

Results: From diarrheatic calves, 51 bacterial isolates were biochemically identified as *E. coli* and 31 isolates out of 51 were considered B₁ phylotype using DNA Microarray technology. Of these isolates, 20 contained *stx_{1a}* and *stx_{1b}* and one harbored all mentioned variants of *stx* genes except *stx_{2b2}*.

Conclusion: This study showed that in Tehran suburb, the B₁ phylotype of *E. coli* is prevalent as a causative agent of diarrhea in calves and the prevalence of *stx₁* gene subtypes is dominant in comparison with other subtypes. Considering the possibility that these *stx* genes can be spread to other strains, bovine *E. coli* strains are an important source of *stx* genes for other strains and further study and surveillance seems to be required for the exact identification of virulence profile of *E. coli* phylotypes in different hosts.

Keywords: *Escherichia coli*, calf diarrhea, B₁ phylotype, shiga-like toxin subtypes, Tehran suburb

INTRODUCTION

Escherichia coli is one of the most important agents causing gastrointestinal tract infection in

meat producing domestic animals, especially at the first weeks of life and ruminants are one of the reservoirs of Shiga like toxin producing *E. coli* (STEC), excreting this infectious agent in feces and environment(1). STEC is a public health threatening germ causing sporadic and outbreaks of human problems including diarrhea, hemorrhagic colitis and Hemolytic-Uremic Syndrome (HUS) characterized by acute renal failure, microangiopathic hemolytic anemia and thrombocytopenia. The ability of STEC

*Corresponding author: Department of Pathobiology, Faculty of Veterinary Medicine, Semnan University, Semnan, Iran.

Telefax: +98233344985

E-mail: hstaji@semnan.ac.ir

to cause these severe complications is related to secretion of Verotoxins which are encoded by *stx*₁ and *stx*₂ genes (2). Direct contact to reservoirs or faecally contaminated foods or water resources are the main transmission routes of STEC to humans (3).

Shiga toxin 1 (*stx*₁) and Shiga toxin 2 (*stx*₂) are encoded on a lambdoid bacteriophage. *stx*₁ is genetically and immunologically distinct from *stx*₂, showing 55–60% genetic and amino acid identity. *stx*₁ is very similar to the Shiga toxin *stx* found in *Shigella dysenteriae* type 1. Despite their similarities, *stx*₁ and *stx*₂ produce different degrees and types of tissue damage. *Enterohemorrhagic E. coli* (EHEC) that produce *stx*₂ are more likely to cause hemolytic uremic syndrome than are *stx*₁ producers (4).

E. coli strains according to the presence of *chuA*, *yjaA* and *TsPE4.C2* are phylogenetically divided into seven groups and subgroups (A₀, A₁, B₁, B₂₂, B₂₃, D₁, and D₂) as follows: subgroup A₀ (group A), lacking *chuA*, *yjaA*, and *TSPE4.C2*; subgroup A₁ (group A), lacking *chuA*, having *yjaA*, and lacking *TSPE4.C2*; subgroup B₂₂ (group B₂), having *chuA* and *yjaA* and lacking *TSPE4.C2*; subgroup B₂₃ (group B₂), having *chuA*, *yjaA*, and *TSPE4.C2*; subgroup D₁ (group D), having *chuA* and lacking *yjaA* and *TSPE4.C2*; and subgroup D₂ (group D), having *chuA*, lacking *yjaA*, and having *TSPE4.C2* (5).

It has been demonstrated that the majority of the *E. coli* strains that are able to persist in the environment belong to the B₁ phylogenetic group (6). Thus, the aim of this study was to identify the prevalence of *E. coli* phylotypes in the cattle farms of Tehran suburbs and estimating their potential to keep the *stx* subtypes in environment as reservoirs.

MATERIALS AND METHODS

Bacterial isolation and identification. Sampling and sample size determination were done according to the table described by Krejcie & Morgan (33). In summary, a total of 140 faecal samples, randomly, from 220 calves (1-15 days old) suffering from diarrhea were collected during January to March (2014) from 460 calves born in dairy herds kept in south east of Tehran as an important region for dairy herds production and *E. coli* isolation was performed according to the protocol described by Alonso et al. (7). Genomic DNA was extracted from isolated strains with the Accu Prep Genomic DNA extraction

kit (BIONEER, Korea) according to the manufacturer's protocol (3).

DNA Labelling. Purified genomic DNA was quantified using a Nanodrop Spectrophotometer (Nanodrop Technologies, Thermo Scientific, USA). Approximately 300 ng of DNA was subjected to fluorescent labelling using the Bioprime DNA labelling system (Invitrogen Life Technologies, Burlington, Canada). Labelling efficiency and the percentage of dye incorporation was then determined by scanning the DNA sample in the Nanodrop spectrophotometer from 200 to 700 nm. Cy3 dye incorporation was calculated using a webbased percent incorporation calculator (available on web page http://www.pangloss.com/seidel/Protocols/percent_inc.html).

Shiga like toxin oligonucleotide microarray. The *E. coli* microarray (maxi-virulence) used in this study was designed and produced by NRC Biotechnology Research Institute (NRC-BRI) and Groupe de Recherchesur le Maladies Infectieuses du Porc (GREMIP). The microarray version used, originally developed by Bruant et al. (8), was composed of 70-mer oligonucleotide probes targeting 264 virulence or virulence-related genes covering all known *E. coli* pathotypes including *stx* probes (Table 1).

Hybridizations and data acquisition. For each hybridization 500 ng of labelled DNA was dried under vacuum in a rotary desiccator without heating (Savant Speed Vac, ArrayIt, USA). Dried labelled DNA was re-suspended in hybridization buffer (DIG Easy Hyb Buffer, Roche Diagnostics, Laval, Canada). Microarrays were pre-hybridized for at least one hour at 50°C with a pre-heated pre-hybridization buffer containing 59 SSC, 0.1% SDS and 1.0% BSA. After pre-hybridization, the microarrays were hybridized with a solution that consisted of 25 µl of hybridization buffer, 20 µl of Bakers Yeast tRNA (10 mg/ml) (Sigma Aldrich, St. Louis, USA) and 20 µl of sonicated Salmon Sperm DNA (10 mg/ml) (Sigma Aldrich), mixed together with the labelled DNA which had previously been denatured. Microarrays were hybridized overnight at 50°C in a SlideBooster (model SB800; Advantix, Germany). After hybridization, stringency washes were performed with Advawash (Advantix) using 19 SSC, 0.02% SDS preheated to 50°C. Microarray slides were scanned with a Scan Array Lite fluorescent microarray

Table 1. The sequence of 70-mer stx gene and phylogenetic marker probes used in the slide array for detection of stx subtypes and phylogenetic groups.

Probe ID.	Oligonucleotide sequence (5→3)	Accession no.
chuA	TTG GCA AGG TGG CAG AAA CAG CTA AGG CCA ATA AAC TCA AAC GCA ACG AGG TAA ATT GCG GAC GTG ACA T	U67920
yjaA	GAT TAC GAC GAA TTT GGA TAT ACA GAA CTG ACA TGA GAT TCC CTT CAT CAT GCA AAT AAT TGA TAT GCA A	AE016770
TSPE4.C2	CTA TCG AAC TTG AAG GGA TGA CCT TAC GAA TAG TGT CAC CGC TGA ATG CCC CGA CAT TAC TCC CGA CGA T	AF222188
stx1A	CAT CCC CGT ACG ACT GAT CCC TGC AAC ACG CTG TAA CGT GGT ATA GCT ACT GTC ACC AGA CAA TGT AAC C	AF461168
stx1B	TCA TCC CCG TAA TTT GCG CAC TGA GAA GAA GAG ACT GAA GAT TCC ATC TGT TGG TAA ATA ATT CTT TAT C	AF461168
stx2A	GTA TTA CCA CTG AAC TCC ATT AAC GCC AGA TAT GAT GAA ACC AGT GAG TGA CGA CTG ATT TGC ATT CCG G	X65949
stx2B-1	AAA TCC GGA GCC TGA TTC ACA GGT ACT GGA TTT GAT TGT GAC AGT CAT TCC TGT CAA CTG AGC ACT TTG C	AE005174
stx2B-2	AAA TCC TGA ACC TGA CGC ACA GGT ATT TGA TTT GAT TGT TAC CGT CAT TCC TGT TAA CTG TGC GCT TTG C	X65949

analysis system (Perkin-Elmer, Mississauga, Canada) using with Scan Array Gx software (Perkin-Elmer, Foster City, USA). Fluorescent spot intensities were quantified with Quant Array Version 3.0 (Packard Bioscience, Boston, USA). All the microarrays were normalized using the same method. For each sub array, the mean value for each set of duplicate spotted oligonucleotides was divided by the correction factor taken from the negative controls spots. This value was then divided by the average of the empty spots to create a signal-to-noise ratio. Oligonucleotide spots with a signal-to-noise fluorescence ratio greater than the established threshold (3 in this case), were considered positive. These ratios were then converted into binary data where a value of 0 indicates a negative probe and a value of 1 a positive probe. A threshold of 3 was chosen because it best represented spot quantification. At least three arrays were hybridized to each strain and the six technical replicate points (two per array) were pooled. At least five probes of the six gene probes had to be positive before a positive score was considered.

RESULTS

According to the biochemical procedure described

by Alonso et al. (7), 51 bacterial isolates were identified as pathogenic *E. coli* from 140 fecal samples.

The 51 *E. coli* strains were phylogenetically grouped based on the presence of *chuA*, *yjaA* and *TSPE4.C2* markers and results demonstrated the distribution of phylotypes in our samples as follow: B₁ (60.78%), D₁ (15.68%), A₀ (9.8%), B₂₃ (5.88%), A₁ (3.9%), B₂₂ (1.9%) and D₂ (1.9%) and B1 phylotype was the most distributed group in our study existing in farms of defined area, causing calf diarrhea. The detection of *stx* gene subtypes in B₁ phylotype, showed that from thirty one B₁ strains, ten (32.2%) strains did not have any *stx* subtypes and twenty one (67.8%) strains harbored at least one subtypes of *stx* toxin genes as follow: twenty (64.5%) with two subtypes (*stx₁A*+*stx₁B*), one (3.3%) strain with four subtypes (*stx₁A*+*stx₁B*+*stx₂A*+*stx₂B₁*) (Table 2).

DISCUSSION

Escherichia coli is an important infectious agent in calves less than 2 month old (9). *E. coli* strains according to the presence of *chuA*, *yjaA* and *TSPE4.C2* are phylogenetically divided into seven groups and subgroups (A₀, A₁, B₁, B₂₂, B₂₃, D₁, and D₂). To increase the discrimination power of *E. coli* population

Table 2. Distribution of phylogenetic groups (FG) among *E. coli* strains from calves with diarrhea and frequency of *stx* subtypes genes in B₁ isolates.

Phylogenetic Groups	No.strains (%)	<i>stx</i> subtype genes (% of <i>stx</i> genes in B ₁ FG)		
		<i>stx</i> ₁ A+ <i>stx</i> ₁ B	<i>stx</i> ₁ A+ <i>stx</i> ₁ B+ <i>stx</i> ₂ A+ <i>stx</i> ₂ b ₁	without <i>stx</i> gene
B ₁	31 (60.78%)	20 strains (64.5%)	1 strain (3.3%)	10 strains (32.2%)
D ₁	8 (15.68%)			
A ₀	5 (9.8%)			
B ₂₃	3 (5.88%)			
A ₁	2 (3.9%)			
B ₂₂	1 (1.9%)			
D ₂	1 (1.9%)			
Total	51 (100%)			

analyses, it has been proposed the use of subgroups that are determined by the combination of the genetic markers (10). Some authors analyzed the distribution of the main phylogenetic groups among *E. coli* strains isolated from human and animal feces. Gordon and Cowling (2003) observed that the relative abundance of phylogenetic groups among mammals is dependent on the host diet, body mass and climate (11). Escobar-Páramo et al. (2006) analyzing fecal strains isolated from birds, non-human mammals and humans, observed the prevalence of groups D and B₁ in birds, A and B₁ in non-human mammals, and A and B₂ in humans (10). These authors concluded that one of the main forces that shape the genetic structure of *E. coli* populations among the hosts is domestication. Baldy-Chudzik et al. (2008) analyzed feces from zoo animals and found a prevalence of group B₁ in herbivorous animals and a prevalence of group A in carnivorous and omnivorous animals (12). In this work we described the distribution of different *E. coli* phylotypes in some cattle farms of Tehran region and we found that B₁ phylotype is the most phylotype causing diarrhea in newborn calves. According to the observation that STEC is quite prevalent in cattle as well has been reported by Pradel et al. (2000) and Kobayashi et al. (2001), who found 70 and 100% of cattle *stx* positive in their respective studies (13, 14). We monitored the presence of different *stx* gene subtypes in the members of B₁ phylotype and the main result is that *stx*₁ subtypes are the most prevalent in isolated strains and only one strain carrying *stx*₁ and *stx*₂ subtypes. Carlos et al. (2010) indicated that distribution of phylogroup

genetic markers amongst the *E. coli* strains associated with mammals are not randomly distributed presenting an average of 96% overlapping and similarity (6). Apajalahti (2005) showed that cows, goats and sheep as ruminant mammals differ from other animals for many gut characteristics and the diet. It has been reviewed that these factors affect phylogroup profile of mammals and it has also been shown that B1 phylogroup is the most prevalent group in herbivorous mammals while the omnivorous animals presented the phylogroup A, dominantly (15). Geographic factors was previously reported to affect the *E. coli* population structure among hosts (6). Although we found B₁ phylotype as the most prevalent group causing diarrhea in newborn calves in Tehran suburb and it is parallel with the results obtained with Apajalahti (15), other investigators reported phylogroup B₂ strains among herbivorous and omnivorous mammals, but found B₁ phylogroup among birds and carnivorous mammals (11). Salehi and Ghanbarpour (2010) did a phylogroup profiling in *E. coli* strains from Japanese quail demonstrating that 50 percent of isolates belong to phylogroup A, the remainders belonged to B₁, B₂ and D groups subsequently (16). Their result is similar to finding of Gordon and Cowling (2003) (11).

There is no data available about the frequency of *stx*₂ and *stx*₁ in animal and people in close contact to HUS patients in Iran. The greater observation of the *stx*₂ gene relative to the *stx*₁ gene in strain populations indicates a risk alert of this gene between these populations. Some studies have revealed that strains possessing only *stx*₂ are potentially more virulent than

strains harboring *stx*₁ or even strains carrying both *stx*₁ and *stx*₂ (17,18). It is of note that most HUS-associated clinically relevant STEC isolates produce *stx*₂, but at least in Europe, rarely, *stx*₁ is highly relevant (17). *Stx*₂ has been found to be approximately 400 times more toxic (as quantified by LD50 in mice) than *Stx*₁ (17, 23). The gene belonging to strains detected from animals showed more expression of protein toxin than human samples (18). Hence the strains of animal origin maintain the characteristic and are more cytotoxic than the gene from human origin (22). This supports the suggestion of Tahamtan et al. (2010) that cattle may have been the source of the organism for the HUS patients (23).

Walk et al. (2007) demonstrated that the majority of the *E. coli* strains that are able to persist in the environment belong to the B₁ phylogenetic group (5). Our data revealed high levels of *stx*₁ gene-carrying bacteria in fecal samples from different cattle. STEC strains among the B₁ group harboring *stx*₁ was isolated more (64.5%) than STEC *stx*₂ (3.3%). Zahraei Salehi et al. (2006) identified STEC O157 among 7 isolates (11.5%), from cattle, whereas non-O157 strains that are frequently associated with sporadic cases of HUS (24, 25), were isolated from 4 (6%) of animals. They showed 5 (8.2%) isolates carried *stx* genes (25). This finding was in parallel with the results of Jomezadeh et al. (2008) that showed the presence of *stx*₁ in 35.5 and *stx*₂ in 49.1% of human isolates (27). This is in contrast with Sepehriseresht et al. (2008) finding with a report of *stx*₁ and *stx*₂, among 5% and 1.9% of calves respectively (28). Zahraei Salehi et al. and Mazhaeri Nejad Fard et al. (2005), reported that prevalence of STEC strains in calves with diarrhea in Tehran, was 68.8% (13.7% of isolates were *stx*₁+ and 55.1% carrying *stx*₂ gene) and 21.8%, respectively (24, 29). In another report, STEC strains were diagnosed in 20.9% of *E. coli* strains from calves with diarrhea in Urmia, West Azerbaijan province (30) while other studies show the prevalence of STEC strains within *E. coli* isolates from calves suffering from diarrhea 26%, 27%, 17.8% and 2.7% in Charmahal, Fars, Khozestan and Isfahan province, respectively (31), while our findings showed that 14.3% of tested calves carrying *stx*₁ positive strains and less than one percent *stx*₂ harboring strains. Our finding is approximately similar to results obtained by Zahraei Salehi et al. (32). This may be as a result of geographical conditions, the presence of natural antibodies and differences in the natural intestinal flora present in humans and animals.

In conclusion, there is no data available about distribution of *E. coli* phylotypes and distribution of *stx* genes within these phylotypes in different regions of Iran. Keeping in mind the members of B₁ phylotype as commensally bacteria and circulation of *stx* genes between them as virulence factors and their ability to transmit these factors vertically and horizontally, more work and comprehensive diagnosis of *E. coli* phylotypes in different hosts and their virulence factors as in detailed epidemiological data, seems to be necessary.

REFERENCES

1. Djordjevic SP, Hornitzky MA, Bailey G, Gill P, Vanselow B, Walker K, et al. Virulence properties and serotypes of Shiga toxin producing *Escherichia coli* from healthy Australian slaughter-age sheep. *J Clin Microbiol* 2001; 39:2017–2021.
2. Kobayashi H, Shimada J, Nakazawa M, Morozumi T, Pohjanvitra T, Pelkonen S, et al. Prevalence and characteristics of Shiga toxin-producing *Escherichia coli* from healthy cattle in Japan. *Appl Environ Microbiol* 2001; 67:484–489.
3. Zahraei Salehi T, Tonelli A, Mazza A, Staji H, Badagliacca P, Ashrafi Tamai I, et al. Genetic characterization of *Escherichia coli* O157:H7 strains isolated from the one-humped camel (*Camelus dromedarius*) by using microarray DNA technology. *Mol Biotechnol* 2012; 51:283–288.
4. Lee JE, Reed J, Shields MS, Spiegel KM, Farrell LD, Sheridan PP. Phylogenetic analysis of Shiga toxin 1 and Shiga toxin 2 genes associated with disease outbreaks. *BMC Microbiology* 2007; 7:109.
5. Gordon D, Clermont O, Tolley H, Denamur E. Assigning *Escherichia coli* strains to phylogenetic groups: multi-locus sequence typing versus the PCR triplex method. *Environ Microbiol* 2008; 10: 2484–2496.
6. Carlos C, Pires MM, Stoppe NC, Hachich EM, IZ Sato M, Gomes TAT. *Escherichia coli* phylogenetic group determination and its application in the identification of the major animal source of fecal contamination. *BMC Microbiology* 2010; 10:161.
7. Alonso JL, Soriano A, Carbajo O, Amoros I, Garelick H. Comparison and recovery of *Escherichia coli* and thermotolerant coliforms in water with a chromogenic medium incubated at 41 and 44.5 C. *Appl and Environ Microbiol* 1999; 65:3746–3749.
8. Bruant G, Maynard C, Bekal S, Gaucher I, Masson L, Brousseau R, et al. Development and validation of

- an oligonucleotide microarray for detection of multiple virulence and antimicrobial resistance genes in *Escherichia coli*. *Appl Environ Microbiology* 2006; 72: 3780–3784.
9. Acha SJ, Kuhn I, Johnsson P, Mbazima G, Kattouli M, Mollby R. Studies on calf diarrhea in Mozambique: Prevalence of bacterial pathogens. *Acta Vet Scand* 2004; 45: 27-36.
 10. Escobar-Páramo P, Le Menac'h A, Le Gall T, Amorin C, Gouriou S, Picard B. Identification of forces shaping the commensal *Escherichia coli* genetic structure by comparing animal and human isolates. *Environ Microbiol* 2006; 8:1975-1984.
 11. Gordon DM, Cowling A. The distribution and genetic structure of *Escherichia coli* in Australian vertebrates: host and geographic effects. *Microbiology* 2003; 149:3575-3586.
 12. Baldy-Chudzik K, Mackiewicz P, Stosik. Phylogenetic background, virulence gene profiles, and genomic diversity in commensal *Escherichia coli* isolated from ten mammal species living in one zoo. *Vet Microbiol* 2008; 131:173-184.
 13. Pradel N, Livrelli V, De Champs C, Palcoux JB, Reynaud A, Scheutz F, et al. Prevalence and characterization of Shiga toxin-producing *Escherichia coli* isolated from cattle, food, and children during a one-year prospective study in France. *Clin Microbiol* 2000; 38: 1023–1031.
 14. Kobayashi H, Shimada J, Nakazawa M, Morozumi T, Pohjanvirta T, Pelkonen S, et al. Prevalence and characteristics of Shiga toxin-producing *Escherichia coli* from healthy cattle in Japan. *Appl Environ Microbiol* 2001; 67: 484–489.
 15. Apajalahti J. Comparative gut microflora, metabolic challenges, and potential opportunities. *J Appl Poult Res* 2005; 14:444-453.
 16. Salehi M, Ghanbarpour R. Phenotypic and genotypic properties of *Escherichia coli* isolated from colisepticemic cases of Japanese quail. *Trop Anim Health Prod* 2010; DOI 10.1007/s11250-010-9583-5.
 17. Ilingsona JLE, Koziczowska JJ, Andersona JL, Carl. sonb SA, Sharmab VK. Rapid PCR detection of enterohemorrhagic *Escherichia coli* (EHEC) in bovine food products and feces. *Mol Cell Probes* 2005; 19: 213-217.
 18. Nataro JP, Kaper JB. Diarrheagenic *Escherichia coli*. *Clin Microbiol Rev* 1998; 11: 142-201.
 19. Ludwig K, Sarkim V, Bitzan M, Karmali MA, Bobrowski C, Ruder H, et al. Shiga Toxin-Producing *Escherichia coli* infection and antibodies against *Stx₂* and *Stx₁* in household contacts of children with enteropathogenic hemolytic-uremic syndrome. *J Clin Microbiol* 2002; 40: 12-17.
 20. Riley LW, Remis RS, Helgerson SD, McGee HB, Wells JG, Davis BR, et al. Hemorrhagic colitis associated with a rare *Escherichia coli* serotype. *New Eng J Med* 1983; 308: 681–685.
 21. Keen EJ, Elder RO. Isolation of Shiga-toxigenic *Escherichia coli* O157 from hide surface and the oral cavity of finished beef feedlot cattle. *JAMA* 2002; 220: 756-763.
 22. Ludwig K, Karmali MA, Sarkim V, Bobrowski C, Petric M, Karch H, et al. Antibody response to Shiga toxins *Stx₂* and *Stx₁* in children with enteropathic hemolytic-uremic syndrome. *J Clin Microbiol* 2001; 39: 2272–2279.
 23. Tahamtan Y, Hayati M, Namavari MM. Prevalence and distribution of the *stx₁*, *stx₂* genes in Shiga toxin producing *E. coli* (STEC) isolates from cattle. *Iran J Microbiol* 2010; 2: 8-13.
 24. Zahraei Salehi T, Mahzounieh M, Asadian F, Khosravi M. Virulence genes in *Escherichia coli* isolates from calves in shahrekord area, Iran. *16th European Congress of Clinical Microbiology and Infectious Disease, Nice, France 2006*.
 25. Rahimi E, Momtaz H, Hemmatzadeh F. The prevalence of *Escherichia coli* O157:H7, *Listeria monocytogenes* and *Campylobacter* spp. on bovine carcasses in Isfahan, Iran. *Iran J Vet Res* 2008; 9: 365-370.
 26. De Sablet T, Bertin Y, Varelle M, Girardeau JP, Garivier A, Gobert AP, et al. Differential expression of *stx₂* variants in Shiga toxin-producing *Escherichia coli* belonging to seropathotypes A and C. *Microbiol* 2008;154: 176–186.
 27. Jomezadeh N, Farajzadeh Sheikh A, Khosravi AD, Amin M. Detection of Shiga toxin producing *E. coli* strains isolated from stool samples of patients with Diarrhea in Abadan Hospitals. *Iran J Biolog Sci* 2009; 9: 820-824.
 28. Sepehriseresht S, Zahraei Salehi T, Sattari M, Tadjbakhsh H, Aslani MM. Detection of shigatoxigenic *Escherichia coli* from fecal samples of calves and cattle by molecular and serological methods. *Comparat-Clin Pathol* 2008; 3: 12-17.
 29. Mazhaherinejadfard R, Behzadiannezhad G, Zahraei Salehi T, Atashparvar N. Evaluation of *ehxA*, *stx₁*, and *stx₂* virulence gene prevalence in cattle *Escherichia Coli* isolates by multiplex PCR. *Arch RaziIns* 2005; 60: 55-66.
 30. Dastmalchi Saei H, Ayremlou N. Characterization of Shiga toxin-producing *Escherichia coli* (STEC) in feces of healthy and diarrheic calves in Urmia region, Iran. *Iran J Microbiol* 2012; 4: 63-69.
 31. Shahrani M, Safarpour Dehkordi F, Momtaz H. Char-

- acterization of *Escherichia coli* virulence genes, pathotypes and antibiotic resistance properties in diarrheic calves in Iran. *Biological Research* 2014; 47:28.
32. Yaghobzadeh N, Ownagh A, Mardani K, Khalili M. Prevalence, molecular characterization and serology of Shiga toxin producing *Escherichia coli* isolated from buffaloes in West Azerbaijan, Iran. *Int J Vet Res* 2011; 2: 113-117.
33. Krejcie RV, Morgan DW. Determining Sample Size for Research Activities. *Educational and Psychological Measurement* 1970; 30: 607-610.