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ORIGINAL ARTICLE

Distribution of Shiga toxin genes *subtypes* in B_1 phylotypes of *Escherichia coli* isolated from calves suffering from diarrhea in Tehran suburb using DNA oligonucleotide arrays

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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 B_1 phylotypes.

Results: From diarrheatic calves, 51 bacterial isolates were biochemically identified as *E. coli* and 31 isolates out of 51 were considered B_1 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_2b_2.

Conclusion: This study showed that in Tehran suburb, the B_1 phylotype of *E. coli* is prevalent as a causative agent of diarrhea in calves and the prevalence of stx_1 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

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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 (A_0, A_1, B_1, B_{22}, B_{23}, D_1, and D_2) as follows: subgroup A_0 (group A), lacking *chuA, yjaA*, and *TSPE4.C2;* subgroup A_1 (group A), lacking *chuA*, having *yjaA*, and lacking *TSPE4.C2;* subgroup B_{22} (group B_2), having *chuA* and *yjaA* and lacking *TSPE4.C2;* subgroup B_{23} (group B_2), having *chuA, yjaA,* and *TSPE4.C2;* subgroup D_1 (group D), having *chuA* and lacking *yjaA* and *TSPE4.C2;* and subgroup D_2 (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 B_1 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 Bruantet 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; Advalytix, Germany). After hybridization, stringency washes were performed with Advawash (Advalytix) using 19 SSC, 0.02% SDS preheated to 50°C. Microarray slides were scanned with a Scan Array Lite fluorescent microarray

Table1. 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: B_1 (60.78%), D_1 (15.68%), A_0 (9.8%), B_{23}(5.88%), A_1 (3.9%), B_{22} (1.9%) and D_2 (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 B_1 phylotype, showed that from thirty one B_1 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 *(stx_1A+stx_1B)*, one (3.3%) strain with four subtypes *(stx_1A+stx_1B+stx_2A+stx_2b_1)* (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 (A_0, A_1, B_1, B_{22}, B_{23}, D_1, and D_2). 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 B_1 isolates.

Phylogenetic Groups	No.strains (%)	*stx* subtype genes (% of *stx* genes in B_1 FG)		
		stx_1A+stx_1B	$stx_1A+stx_1B+stx_2A+stx_2b_1$	without *stx* 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 B_1 in birds, A and B_1 in non-human mammals, and A and B_2 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 B_1 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 B_1 phylogroup 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 B_1 phylogroup 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). Ggeographic factors was previously reported to affect the *E. coli* population structure among hosts (6). Although we found B_1 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 B_2 strains among herbivorous and omnivorous mammals, but found B_1 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 B_1, B_2 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 B_1 phylogenetic group (5). Our data revealed high levels of stx_1 gene-carrying bacteria in fecal samples from different cattle. STEC strains among the B_1 group harboring stx_1 was isolated more (64.5%) than STEC stx_2(3.3%). Zahraee 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 Mazhaheri 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 Azerbayejan 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 B_1 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.

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