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

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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 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 stx₁a and stx₁b and one harbored all mentioned variants of stx genes except stx₂b.

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
to cause these severe complications is related to secretion of Verotoxins which are encoded by \( stx \) and \( stx \) 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 \textit{Shigella dysenteriae} type 1. Despite their similarities, \( stx_1 \) and \( stx_2 \) produce different degrees and types of tissue damage. \textit{Enterohemorrhagic} \textit{E. coli} (EHEC) that produce \( stx_1 \) are more likely to cause hemolytic uremic syndrome than are \( stx_2 \) producers (4).

\textit{E. coli} strains according to the presence of \textit{chuA}, \textit{yjaA} and \textit{TSPE4.C2} are phylogenetically divided into seven groups and subgroups (\textit{A}, \textit{A}, \textit{B}, \textit{B}, \textit{B}, \textit{D}, and \textit{D}) as follows: subgroup \textit{A}, (group \textit{A}), lacking \textit{chuA}, \textit{yjaA}, and \textit{TSPE4.C2}; subgroup \textit{A}, (group \textit{A}), lacking \textit{chuA}, having \textit{yjaA}, and lacking \textit{TSPE4.C2}; subgroup \textit{B}, (group \textit{B}), having \textit{chuA} and \textit{yjaA} and lacking \textit{TSPE4.C2}; subgroup \textit{B}, (group \textit{B}), having \textit{chuA}, \textit{yjaA}, and \textit{TSPE4.C2}; subgroup \textit{D}, (group \textit{D}), having \textit{chuA} and lacking \textit{yjaA} and \textit{TSPE4.C2}; and subgroup \textit{D}, (group \textit{D}), having \textit{chuA}, lacking \textit{yjaA}, and having \textit{TSPE4.C2} (5).

It has been demonstrated that the majority of the \textit{E. coli} strains that are able to persist in the environment belong to the \textit{B} phylogenetic group (6). Thus, the aim of this study was to identify the prevalence of \textit{E. coli} phylootypes in the cattle farms of Tehran suburbs and estimating their potential to keep the \textit{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 \textit{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/seedl/Protocols/percent_inc.html).

**Shiga like toxin oligonucleotide microarray.** The \textit{E. coli} microarray (maxi-virulence) used in this study was designed and produced by NRC Biotechnology Research Institute (NRC-BRI) and Groupe de Recherches sur 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 \textit{E. coli} pathotypes including \textit{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 \( \mu \)l of hybridization buffer, 20 \( \mu \)l of Bakers Yeast tRNA (10 mg/ml) (Sigma Aldrich, St. Louis, USA) and 20 \( \mu \)l 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.
analysis system (Perkin-Elmer, Mississauga, Canada) using 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+stx2B) (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...
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 B₂ 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 phylogroup is the most prevalent group 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 phylogroup and the main result is that stx₁ subtypes are the most prevalent in isolated strains and only one strain carrying stx₁ and stx₂ 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 phylogroup 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 remainsders 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 stx₁ and stx₂ in animal and people in close contact to HUS patients in Iran. The greater observation of the stx₂ gene relative to the stx₁ gene in strains populations indicates a risk alert of this gene between these populations. Some studies have revealed that strains possessing only stx₁ are potentially more virulent than

| Phylogenetic Groups | No. strains (%) | stx₁ and stx₂ genes (%) of stx genes in B₁ FG | stx₁, A+stx₂, B | stx₁, A+stx₂, B+stx₂, A+stx₂, B₁ | without stx gene |
|---------------------|----------------|-----------------------------------------------|-----------------|----------------------------------|-----------------|
| B₁                  | 31 (60.78%)    |                                               | 20 strains (64.5%) | 1 strain (3.3%) | 10 strains (32.2%) |
| D₁                  | 8 (15.68%)     |                                               |                 |                    |                  |
| A₀                  | 5 (9.8%)       |                                               |                 |                    |                  |
| B₂₂                 | 3 (5.88%)      |                                               |                 |                    |                  |
| A₁                  | 2 (3.9%)       |                                               |                 |                    |                  |
| B₂₂                 | 1 (1.9%)       |                                               |                 |                    |                  |
| D₁                  | 1 (1.9%)       |                                               |                 |                    |                  |
| Total               | 51 (100%)      |                                               |                 |                    |                  |

Table 2. Distribution of phylogenetic groups (FG) among E. coli strains from calves with diarrhea and frequency of stx subtypes genes in B₁ isolates.
strains harboring \textit{stx}_1 or even strains carrying both \textit{stx}_1 and \textit{stx}_2 (17,18). It is of note that most HUS-associated clinically relevant STEC isolates produce \textit{stx}_2, but at least in Europe, rarely, \textit{stx}_1 is highly relevant (17). \textit{Stx}_1 has been found to be approximately 400 times more toxic (as quantified by LD50 in mice) than \textit{Stx}_2 (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 \textit{E. coli} strains that are able to persist in the environment belong to the B1 phylogenetic group (5). Our data revealed high levels of \textit{stx}, gene-carrying bacteria in fecal samples from different cattle. STEC strains among the B_1 group harboring \textit{stx}_1 was isolated more (64.5%) than STEC \textit{stx}_2 (3.3%). Zahraee Saleshi 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 \textit{stx} genes (25). This finding was in parallel with the results of Jomezadeh et al. (2008) that showed the presence of \textit{stx}, in 35.5 and \textit{stx}, in 49.1% of human isolates (27). This is in contrast with Sepehriseresht et al. (2008) finding with a report of \textit{stx}, and \textit{stx}, 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 \textit{stx}, + and 55.1% carrying \textit{stx}, gene) and 21.8%, respectively (24, 29). In another report, STEC strains were diagnosed in 20.9% of \textit{E. coli} strains from calves with diarrhea in Urmia, West Azerbaijan province (30) while other studies show the prevalence of STEC strains within \textit{E. coli} isolates from calves suffering from diarrhea 26%, 27%, 17.8% and 2.7% in Charmahal, Fars, Khoezestan and Isfahan province, respectively (31), while our findings showed that 14.3% of tested calves carrying \textit{stx}, positive strains and less than one percent \textit{stx}, 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 \textit{E. coli} phylogenotypes and distribution of \textit{stx} genes within these phylogenotypes in different regions of Iran. Keeping in mind the members of B_1 phylogotype as commensally bacteria and circulation of \textit{stx} genes between them as virulence factors and their ability to transmit these factors vertically and horizontally, more work and comprehensive diagnosis of \textit{E. coli} phylogenotypes in different hosts and their virulence factors as in detailed epidemiological data, seems to be necessary.

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