Phylogenetic group determination of faecal *Escherichia coli* and comparative analysis among different hosts

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Summary

Phylogenetic analysis has shown that *Escherichia coli* is composed of four main phylogenetic groups (A, B1, B2 and D). Characterization of phylogenetic groups is of clinical interest, as group A and B1 generally associated with commensals, whereas most enteropathogenic isolates are assigned to group D, and group B2 is associated with extra-intestinal pathotype. One hundred *E. coli* strains recovered from faecal samples of dog, chicken, ruminants (sheep, goat and cattle) and human were subjected to phylogenetic analysis based on triplex PCR method, according to a combination of three genetic markers *chuA*, *yjaA* and DNA fragment TspE4.C2. The majority of collected isolates belonged to group D (44%), followed by groups A (32%), and B2 (24%). By sample origin, groups D, A, and B2 were prevalent in 16.7, 50, and 33.3%, respectively for dog isolates; 52.8, 36.1, and 11.1% for chicken isolates; 41.2, 29.4, and 29.4% for ruminants isolates; and 60.9, 8.7, and 30.4% for human isolates, respectively and none of the strains among all the analysed hosts belong to group B1. This study suggests there was a significant difference in the *E. coli* phylogenetic groups, subgroups and genetic markers among the different hosts analysed.

Key words: *Escherichia coli*, Phylogenetic grouping, Triplex PCR

Introduction

Escherichia coli is a normal inhabitant of the gastrointestinal tract of humans and animals. The *E. coli* species have two characteristics encompassing both commensal and intestinal or extra-intestinal pathogenic strains that cause several diseases including diarrhea, urinary tract infections and meningitis (Russo and Johnson, 2003). Some strains are known to cause serious morbidity and mortality and of having been and still being the most thoroughly studied bacterial species (Sabarinath *et al*., 2011).

Four main phylogenetic groups, A, B1, B2 and D, were described by Herzer *et al*. (1990) using multilocus enzyme electrophoresis with the 72 strains of the *E. coli* reference (ECOR) collection (Ochman and Selander, 1984). This finding was subsequently confirmed by Desjardins *et al*. (1995), by comparison of several genetic markers. The assignment of *E. coli* clones to one of these four groups is the basis of phylogenetic studies of the species. Actually, phylogenetic grouping can be done by multilocus enzyme electrophoresis or ribotyping (Clermont *et al*., 2000), but both of these reference techniques are complex and time-consuming and also require a collection of typed strains. In 2000, Clermont *et al*. described a simple approach based on a triplex PCR detecting the genes *chuA* (a gene required for heme transport in enterohemorrhagic *E. coli* O157:H7) and *yjaA* (a gene initially identified in the recent complete genome sequence of *E. coli* K-12, the function of which is unknown) and the anonymous DNA fragment TSPE4.C2. This method assigns the phylogenetic group in approximately 85-90% of the cases (Gordon *et al*., 2008), and the results were strongly correlated with those obtained by multilocus enzyme electrophoresis and ribotyping methods (Clermont *et al*., 2000).

According to Lecointre *et al*. (1998), groups A and B1 are sister groups whereas group B2 is included in an ancestral branch. These phylo-groups apparently differ in their ecological niches, life-history (Gordon and Cowling, 2003) and some characteristics, such as their ability to exploit different sugar sources; their antibiotic-resistance profiles and their growth rate (Gordon, 2004). 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. Furthermore, genome size differs among these phylo-groups, with A and B1 strains having smaller genomes than B2 or D strains (Bergthorsson and Ochman, 1998). Johnson *et al*. (2001) found that strains from phylo-groups B2 and D contained more virulence factors than strains from the phylo-groups A and B1.

Characterization of phylogenetic groups is of clinical interest, as group A and B1 are generally associated with commensals whereas most enteropathogenic isolates are assigned to group D, and group B2 is associated with extra-intestinal pathotypes (Sabarinath *et al*., 2011). To date, there have been very few published studies on phylogenetic grouping of *E. coli* in Iran (Ghanbarpour and Oswald, 2010), and none on comparison of *E. coli* phylogenetic distribution between different species. Therefore, the aim of this study was to determine and compare the distribution of phylogenetic groups in *E. coli* isolated from faeces of different animal species

including dog, chicken, ruminants and human.

Materials and Methods

Isolation and identification of *E. coli*

A total of 100 *E. coli* was isolated from faecal samples of a variety of hosts [dog (24), chicken (36), ruminants (sheep, goat and cattle) (17), and human (23)]. Typical colonies were streaked on EMB agar (Merck-Germany). Typical *E. coli* colonies (with metallic green) were tested for lactose fermentation, oxidase test, citrate utilization, L-lysine decarboxylase, motility, glucose and sucrose fermentation, indole production, tryptophan deamination, hydrogen sulfide production and urea hydrolysis. Isolated strain which exhibited a biochemical profile for *E. coli* were grown in LB broth (Merck-Germany), and stored in a 25% glycerol solution at -70°C until used.

DNA extraction

DNA template preparation was performed by the boiling method as follows: a few colonies were resuspended in 250 µl sterile distilled water. The cells were lysed by heating at 95°C for 10 min. After heating, they were put immediately on ice for 5 min. The supernatant was harvested by centrifugation at 12,000 rpm for 5 min, then transferred to fresh centrifuge tubes and kept at -20°C. The supernatant was used as a source of template for amplification (Abdallah *et al*., 2011).

Triplex PCR

Phylogenetic grouping of the 100 *E. coli* isolates was assessed by a previously reported triplex PCR-based assay (Clermont *et al*., 2000; Gordon *et al*., 2008). All amplification procedures were repeated at least three times. The primer pairs used for PCR amplification were: ChuA.1 (5′-GACGAACCAACGGTCAGGAT-3′) and ChuA.2 (5′-TGCCGCCAGTACCAAAGACA-3′), YjaA.1 (5′-TGAAGTGTCAGGAGACGCTG-3′) and YjaA.2 (5′-ATGGAGAATGCGTTCCTCAAC-3′), and TspE4.C2.1 (5′-GAGTAATGTCGGGGCATTCA-3′) and TspE4.C2.2 (5′-CGCGCCAACAAAGTATTACG-3′), which generate 279-, 211-, and 152-bp fragments, respectively. Briefly, the amplifications were carried out in a total volume of 25 µl, each reaction mixture contained 11.25 µl of distilled H_2O, 2.5 µl of 10X buffer (supplied with *Taq* polymerase) (CinnaGen Co., Iran), 0.75 µl of $MgCl_2$ (CinnaGen Co., Iran), 1 µl of dNTPs (each deoxynucleoside triphosphate at a concentration of 200 mM) (CinnaGen Co., Iran), 1 µl of each primer (20 pmol) (CinnaGen Co., Iran), 2.5 U of *Taq* polymerase (CinnaGen Co., Iran) and 3 µl of DNA template. A negative control (reaction lacking the template DNA) was included in all amplifications performed. Thermal cycler (MJ Mini, BIO-RAD-USA) conditions were as follows: 4 min of initial denaturation at 94°C followed by 30 cycles of 5-sec denaturation at 94°C; 10 sec of annealing at 57°C and a final extension step of 5 min at 72°C. The amplification products were separated in 2% agarose gels containing ethidium bromide. After electrophoresis, the gel was photographed under UV light. The results allowed the classification of isolates into one of the four major phylogroups (A, B1, B2, or D) (Abdallah *et al*., 2011). Strains were assigned to phylogenetic groups on the basis of presence or absence of the 3 DNA fragments: *chuA*–, TspE4.C2–, group A; *chuA*–, *yjaA*–, TspE4.C2+, group B1; *chuA*+, *yjaA*+, group B2; *chuA*+, *yjaA*–, group D. Because 2 possible profiles can be obtained for the groups A, B2, and D, each was subdivided as follows: *chuA*–, *yjaA*–, TspE4.C2–, group A subgroup A_0; *chuA*–, *yjaA*+, TspE4.C2–, group A subgroup A_1; *chuA*+, *yjaA*+, TspE4.C2–, group B2 subgroup $B2_2$; *chuA*+, *yjaA*+, TspE4.C2+, group B2 subgroup $B2_3$; *chuA*+, *yjaA*–, TspE4.C2–, group D subgroup D_1; *chuA*+, *yjaA*–, TspE4.C2+, group D subgroup D_2 (Gordon *et al*., 2008) (Fig. 1).

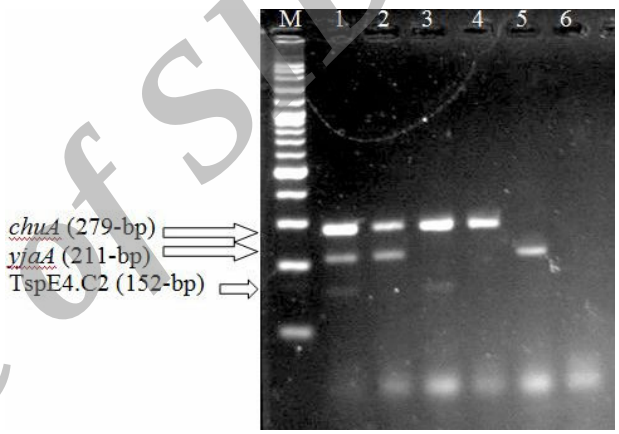

Fig. 1: Triplex PCR based phylogenetic profile of *E. coli* isolates. Lane M: 100 bp marker. Lane 1: Subgroup $B2_3$, Lane 2: Subgroup $B2_2$, Lane 3: Subgroup D_2, Lane 4: Subgroup D_1, Lane 5: Subgroup A_1, and Lane 6: Subgroup A_0

Statistical analysis

The associations between phylogenetic groups, phylogenetic subgroups, genetic markers and hosts (dog, chicken, ruminants and human) were assessed by means of contingency Chi-squares (X^2-test) performed with the SPSS ver. 16.0 (SPSS Inc., Chicago, IL, USA) software.

Results

A total of 100 *E. coli* strains isolated from faeces of different animals and humans were allocated into three phylogenetic groups (i.e. A, B2 and D) and six subgroups (i.e. A_0, A_1, $B2_2$, $B2_3$, D_1 and D_2). According to multiplex PCR-based phylotyping, group D contained the majority of collected isolates (44 isolates, 44%), followed by groups A (32 isolates, 32%), and B2 (24 isolates, 24%). By sample origin, groups D, A, and B2 were prevalent in 16.7, 50, and 33.3%, respectively for dog isolates; 52.8, 36.1, and 11.1% for chicken isolates; 41.2, 29.4, and 29.4% for ruminant isolates; and 60.9, 8.7, and 30.4% for human isolates, respectively. It is interesting to note that strains from group B1 were not found among all the analysed hosts.

Table 1: Distribution of the *E. coli* phylogenetic subgroups among the hosts analysed

Phylogenetic subgroup	Dog	Chicken	Ruminants	Human	Total
A_0	11 (45.8%)	13 (36.1%)	0 (0%)	0 (0%)	24 (24%)
A_1	1 (4.2%)	0 (0%)	5 (29.4%)	2 (8.7%)	8 (8%)
B1	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
$B2_2$	3 (12.5%)	4 (11.1%)	1 (5.9%)	3 (13%)	11 (11%)
$B2_3$	5 (20.8%)	0 (0%)	4 (23.5%)	4 (17.4%)	13 (13%)
D_1	4 (16.7%)	18 (50%)	2 (11.8%)	11 (47.8%)	35 (35%)
D_2	0 (0%)	1 (2.8%)	5 (29.4%)	3 (13%)	9 (9%)
Total	24 (100%)	36 (100%)	17 (100%)	23 (100%)	100 (100%)

Table 2: Distribution of the *E. coli* genetic markers among the hosts analysed

Genetic marker	Dog	Chicken	Ruminants	Human	Total
chuA	12 (17.6%)	23 (33.8%)	12 (17.6%)	21 (30.9%)	68 (100%)
yjaA	9 (28.1%)	4 (12.5%)	10 (31.3%)	9 (28.1%)	32 (100%)
TspE4.C2	5 (22.7%)	1 (4.5%)	9 (40.9%)	7 (31.8)	22 (100%)

Most of the isolated strains from dog samples were included in subgroup A_0, that is, these strains did not reveal the presence of the genetic markers investigated, and none of the dog strains belong to subgroup D_2. Most of the strains of chickens and humans fell within group D_1, and none of these strains belong to subgroup A_1, $B2_3$ and A_0, respectively. In ruminants isolates, the majority of strains were in subgroup A_1 and D_2, and no strain was in subgroup A_0 (Table 1).

A Chi-square value of 16.750, 6 degrees of freedom (D.F.), $P<0.05$, was obtained from a contingency table with the phylogenetic groups distribution among the hosts, allowing the null hypothesis, which states that there is no association between the hosts and the groups, to be rejected (P=0.01). This result suggests a significant difference in the *E. coli* population structure among the animals analysed.

A Chi-square test at the subgroup level was performed to verify the existence of an association between the hosts and the phylogenetic subgroup. The calculated 58.108 Chi-square value (15 D.F.) led to the rejection of the null hypothesis ($P<0.001$).

Based on Clermont method, the presence of *chuA* denotes a strain belonging to phylo-group B2 or D.

The gene *yjaA* distinguishes phylo-group B2 from phlyo-group D strains and is present in most of the phylogroup A strains. The TSPE4.C2 fragment is present in all phylo-group B1 strains, most of the phylo-group B2 strains and few of the phylo-group D strains (Gordon and Cowling, 2003).

The majority of *chuA*, *yjaA* and TspE4.C2 genetic markers were in chicken, ruminant and ruminant, respectively. A Chi-square test was also performed to verify the association between the hosts and the genetic markers (*chuA*, *yjaA* and TspE4.C2). The result (Chi-square value = 13.615, 6 D.F., $P<0.05$) indicated that the genetic markers are distributed differently among the hosts (Table 2).

Discussion

Knowledge of the structure of bacterial populations is a prerequisite to the understanding of the epidemiology of infectious diseases. For the first time in Iran, we determined and compared the distribution of phylogenetic groups in 100 *E. coli* isolated from faeces of dog, chicken, ruminants and human. *Escherichia coli* strains were allocated into three phylogenetic groups of A, B2 and D, and six subgroups of A_0, A_1, $B2_2$, $B2_3$, D_1 and D_2. Among all *E. coli*, except dog isolates, most strains belong to group D. In dog isolates the majority of the strains belong to group A followed by group B2 and D, respectively. The pattern of proportion of phylogenetic groups in chicken, ruminants and human were group D > group A > group B2; group D > group A = group B2, and group D > group B2 > group A, respectively.

Escherichia coli strains belonging to group B2 are highly pathogenic and frequently responsible for extraintestinal infections in humans (Lecointre *et al*., 1998; Duriez *et al*., 2001). *Escherichia coli* strains from group D have fewer virulence determinants than strains from group B2 (Lecointre *et al*., 1998). Extraintestinal pathogenic *E. coli* can be found in group D (Picard *et al*., 1999) and according to Clermont *et al*. (2000), *E. coli O157:H7* could belong to this phylogenetic group (Also named Phylo-group E which exhibit the + -- Clermont phylotype). Thus, a great deal can be learnt concerning the characteristics of an unknown strain by determining its phylo-group membership. Several studies showed the distribution of the main phylogenetic groups among *E. coli* strains isolated from different origins including human and animal faeces. One of them revealed that the relative abundance of phylogenetic groups among mammals is dependent on the host diet, body mass and climate (Gordon and Cowling, 2003). Escobar-Páramo *et al*. (2006) observed the prevalence of groups D and B1 in birds, A and B1 in non-human mammals, and A and B2 in humans by analysing faecal strains isolated from birds, non-human mammals and humans. The other study analysed faeces from zoo animals and found a prevalence of group B1 in herbivorous animals and a prevalence of group A in carnivorous and omnivorous animals (Baldy-Chudzik *et al*., 2008). In healthy food-producing animals, a predominant distribution of group B1 was reported in *E. coli* from cattle, while group A

was predominantly prevalent in pigs and chickens (Escobar-Páramo *et al*., 2006; Carlos *et al*., 2010). The phylogenetic group A was commonly predominant in isolates from cattle and pigs whereas groups A and D were predominant in isolates from poultry. The B2 group was rarely found in *E. coli* from healthy cattle, chickens and pigs in Brazil (Carlos *et al*., 2010) as well as Japan. In Korea, group B2 was also not found in *E. coli* isolates from food-producing animals (Unno *et al*., 2009). Groups A and D were predominant in *E. coli* from diseased poultry and group B2 was also found in 19% (Rodriguez-Siek *et al*., 2005). In European countries, group B2 was often isolated from diseased poultry (Ewers *et al*., 2009; Mora *et al*., 2009). Harada *et al*. (2012) revealed that group B2 was the most prevalent phylogenetic group in canine faecal isolates. Bukh *et al*. (2009) showed that two-thirds of 1533 *E. coli* isolates in Danish patients with community-acquired bacteraemia (CAB) were classified into phylogenetic group B2. Groups A and D were comparable in size, whereas B1 was the least abundant. Thus, the distribution of phylogenetic groups may be determined not only by the animal species but also by their health status or geographical region, and these differences in the distribution of the phylogenetic groups among the strains of this study and other similar studies may be due to three main factors: (i) geographic climatic conditions, (ii) dietary factors and/or the use of antibiotics, or (iii) host genetic factors; some *E. coli* strains may be primarily adapted to the gut conditions of certain populations (Duriez *et al*., 2001).

Carlos *et al*. (2010) reported that the *chuA* and *yjaA* genes were rarely found in isolated strains of cows, goats and sheep but were commonly found in human, chicken and pig strains. Sobieszczaeska (2008) showed that 95.5% of the enteroaggregative *E. coli* strains carried the *chuA* gene, which encodes for a haem receptor. Our study demonstrated that phylogenetic subgroup, group and genetic markers distribution are not randomly distributed among the hosts analysed, and there are associations between them.

In conclusion, our study suggests that the distribution of phylogenetic groups in *E. coli* from different animals varies regionally in addition to animal species. The results obtained in this work suggest that PCR-based methods, applied to identify the phylogenetic groups A, B1, B2 and D, can be used for a rapid assessment and are relatively inexpensive, highly reproducible typing tests for epidemiological studies of *E. coli* in different hosts. This would be helpful as an initial screening assay, given the established link between phylogenetic group and virulence (Clermont *et al*., 2000), and could be used to complement more time consuming traditional tests that use serological and animal assays. This test is simple, reproducible and accessible to most laboratories with limited resources.

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مطالعات فیلوژنی نشان داده‌اند که باکتری *اشریشیا کلی* (*E. coli*) از چهار گروه اصلی فیلوژنی (A، B1، B2 و D) تشکیل شده است. تعیین گروه‌های فیلوژنی دارای اهمیت کلینیکی می‌باشد چنان که گروه A و B1 عموما در ارتباط با سویه‌های کمنسال بوده در حالی که بیشتر جدایه‌های بیماریزای روده‌ای در گروه D طبقه بندی می‌شوند و گروه B2 مربوط به سویه‌های بیماریزای خارج روده‌ای می‌باشد. تعداد 100 سویه باکتری *اشریشیا کلی* جدا شده از نمونه‌های مدفوع سگ، طیور، نشخوارکنندگان (گوسفند، بز و گاو) و انسان تحت مطالعه فیلوژنی بر اساس روش triplex PCR و با استفاده از ترکیب سه مارکر ژنتیکی *chuA*، *yjaA* و قطعه TspE4.C2 از DNA قرار گرفت. بیشتر جدایه‌های جمع آوری شده متعلق به گروه D (44٪) و سپس گروه A (32٪) و B2 (24٪) بودند. بر اساس منشاء نمونه، گروه‌های D، A و B2 به ترتیب دارای شیوع 16/7٪، 50٪ و 33/3٪ برای جدایه‌های سگ، 52/8٪، 36/1٪ و 11/1٪ برای جدایه‌های طیور، 41/2٪، 29/4٪ و 29/4٪ برای جدایه‌های نشخوارکنندگان و 60/9٪، 8/7٪ و 30/4٪ برای جدایه‌های انسان بوده و هیچ کدام از سویه‌ها در میزبان‌های مورد مطالعه متعلق به گروه B1 نبوده‌اند. این مطالعه نشانگر وجود تفاوت مشخص در گروه‌ها و زیرگروه‌های فیلوژنی و مارکرهای ژنتیکی باکتری *اشریشیا کلی* در بین میزبان‌های مختلف مورد مطالعه می‌باشد.

واژه‌های کلیدی: *اشریشیا کلی*، گروه بندی فیلوژنی، Triplex PCR