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Molecular characterization, genetic diversity, and antibacterial susceptibility of *Escherichia coli* encoding Shiga toxin 2f in domestic pigeons

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Running title: Characterization, diversity and susceptibility of Stx2f - STEC

Significance and Impact of the Study: Carriage of *stx2f* gene tends to be underreported in pigeon *Escherichia coli* isolates because most routine genetic and phenotypic tests cannot efficiently target this gene or detect the toxin. Nevertheless, pigeons frequently carry *E. coli* strains that are *stx2f*-positive and this situation is not limited to any distinct geographical area. The current results suggest that genetic background of *stx2f*-encoding *E. coli* is distinct from most Shiga-toxin producing *E. coli* strains. However, the factors that contribute to host preferences and pathogenicity remain unclear. These findings have public health significance that should be addressed in future research.

Abstract

This study aimed to evaluate prevalence, characteristics, genotypic diversity, and antibacterial susceptibility of *Escherichia coli* encoding Shiga toxin 2f in domestic pigeons in different provinces of Iran. A total of 117 fecal samples were collected from pigeons, and were subjected to molecular detection of *stx2f*. In total, 20%, 25.8%, 21.4% and 9% of pigeons from Tehran, Ferdows, Garmsar and Babol cities carried *stx2f*+ isolates respectively. Of the 460 *E. coli* isolates examined, 43 were *stx2f*+ and most also carried *eae* (95.3%) and *astA* (97.7%) genes. Some of the *stx2f*+ isolates harboured *cnf* (9.3%), but all were negative for *stx1*, *stx2* (other subtypes) and *ehly*. Most Strains (90%) were assigned to B1 phylogroup and possessed Intimin-β. Fingerprinting of the *stx2f*+ isolates using either enterobacterial repetitive intergenic consensus sequences (ERIC) or random amplified polymorphic DNA (RAPD) – polymerase chain reaction revealed 7 distinct profiles by each method, with one prevailing (65% and 39.5%, respectively). By the combination of methods 10 profiles were recognised. Ten isolates from different profiles were shown to belong to O20, O78, and O115 serogroups, and eight were 100% identical in the *stx2f* gene sequence. The strains were consistently resistant to amoxicillin and lincospectin, and commonly resistance to tetracycline (88.4%) and doxycycline

(77.4%). Overall, the results indicate a limited degree of genetic diversity in *stx2f*-harbouring *E. coli* from pigeons.

Keywords: *E. coli*, Stx2f, Molecular epidemiology, Virulence

Introduction

Shiga toxin-producing *Escherichia coli* (STEC) is responsible for a number of infectious diseases in humans and animals ranging from mild diarrhea to life threatening haemolytic uremic syndrome (HUS). The pathogenic capacity of STEC strains resides in a number of virulence factors, with Shiga toxins (Stx) being the most important. Two types of Shiga toxins have been described: Stx1, which is rather homologous, and Stx2 which is more heterogeneous in nature and consists of seven known subtypes including Stx2f (Mainil and Daube 2005). Since the first isolation of Stx2f-producing *E. coli* from a diarrheic patient (Gannon *et al*. 1990) (the relevant toxin was initially designated as SltIIva), the organism has been isolated from pigeons and some cases of human infections (Schmidt *et al.* 2000, Morabito *et al.* 2001, Farooq *et al*. 2009, Prager *et al.* 2009). Most STEC strains have evolved with rich repertoire of virulence determinants. One of the major virulence factors is intimin, an outer membrane protein that mediates intimate attachment to intestinal epithelial cells. The C-terminal end of intimin protein (Int-280) is highly variable, allowing the recognition of intimin subtypes. It is also the cell-binding domain of intimin , which can affect pathogen-host interactions (Mainil and Daube 2005).

Although Stx2f-producing *E. coli* is not generally regarded as a highly pathogenic STEC, recent studies show increasing evidence links to human infections especially infantile diarrhea (Ethoh *et al.* 2009; Prager *et al.* 2009). Presence of strains harbouring *stx2f* tend to be underreported in both humans and animals, partly because most of the available molecular and serological diagnostic methods produce unreliable results in the detection of the *stx2f* gene or its product (Schmidt *et al.* 2000; Ethoh *et al.* 2009; Prager *et al.* 2009; Feng *et al*. 2011). Infrequent identification of strains possessing *stx2f* has been resulted in limited information on virulence, epidemiology, and antibacterial susceptibility of these strains compared to other STECs.

Domestic pigeons (*Columba livia domestica*) are widespread in different cities, inhabit crowded areas like shrines, and are commonly raised by fanciers in Iran. Therefore, pigeons carrying STEC can be a potential reservoir for pathogenic *E. coli* strains in urban and rural areas. The presence of other *stx* subtypes was previously reported in *E. coli* isolates from pigeons in Iran (Ghanbarpour and Daneshdoost 2012), but the occurrence of *stx2f* harbouring *E. coli* has not been reported. The present study aimed to investigate the prevalence, characteristics, clonal diversity, and antibacterial susceptibility of *stx2f*+ *E. coli* strains isolated from domestic pigeons in four provinces in Iran. Unraveling the genotypic and phenotypic characteristics of STEC strains originated from diverse geographical areas could potentially help better understanding of the epidemiology of associated disease outbreaks, and the mechanisms underlying the evolution of emerging clones.

Results and Discussion

Prevalence and molecular characterization

Among 460 isolates tested, 43 isolates from 22 (18.8%) pigeons included the *stx2f* gene and were regarded as STEC. In summary, 20%, 25.8%, 21.4% and 9% of sampled pigeons from Tehran, Ferdows, Garmsar and Babol carried *stx2f*+ isolates respectively (Table 1). In the multiplex-PCR assay, 41 isolates (95.3%) out of these 43 also harboured the *eae* gene, but none of the isolates contained *stx1*, *stx2* and *ehly*. Screening for other virulence genes revealed the presence of *astA* in 97.7% (42/43), and *cnf* in 9.3% (4/43) of the isolates, but all were negative for *saa*. Subtyping of *eae* gene in 11 isolates showed that 10 isolates contained Int-β, and one Int-α2.

Although pigeons are considered as natural reservoirs of Stx2f-producing *E. coli*, several studies have applied inappropriate methods for detection of STEC strains in pigeons. For example, the Premier EHEC ELISA kit (Meridian Bio-science) and the PCR protocol of Paton and Paton (1998), used by Wani *et al.* (2004), Pedersen *et al.* (2006), Silva *et al*. (2009) and Ghanbarpour and Daneshdoost (2012), both known to be incapable of detecting the *stx2f* subtype (Ziebell *et al*. 2002; Feng *et al.* 2011). This lack of sensitivity for detection of *stx2f* can explain, at least in part, the lower prevalence of STEC strains (2.9% *stx2* and 1.4%

stx1) in pigeons of southeast Iran in comparison to our findings (Ghanbarpour and Daneshdoost 2012). The overall prevalence of *stx2f* –harboring strains in pigeon populations was 18.8%, ranging from 9% to 25.8% in the four studied cities in the current study. Studying pigeons in Germany, Grossmann *et al*. (2005) have found a higher prevalence of *stx2f* in urban areas comparing to other *stx* subtypes. Similar to the findings of the current study, Morabito *et al*. (2001) have reported a prevalence of *stx2f*+ strains of 8%, 12.1%, and 12.4% in pigeons in three squares in Rome. The higher prevalence of STEC in the current study may have resulted from a different screening approach for the identification of *stx2f*+ strains. In contrast, a very low prevalence of *stx2f*+ *E. coli* has been reported in pigeon populations of Finland and Japan (Kobayashi *et al*. 2002; Kobayashi *et al*. 2009).

The results of the current study and previous investigations indicate some unique characteristics of Stx2f-producing *Escherichia coli* strains. First, these strains only harbour one type of Shiga toxin gene; this particular feature is relatively uncommon among other STEC strains (Mainil and Daube 2005; Prager *et al.* 2009). Another important characteristic is the marked consistency with regard to the possession of virulence genes and *eae* subtypes, as almost all isolates in the current study possessed the *eae* and *astA* genes. Similarly, the widespread occurrence of *eae* (mostly the Int-β subtype), *astA,* and *cdt* gene were reported previously in *stx2f*+ strains (Morabito *et al.* 2001; Ethoh *et al.* 2009; Prager *et al*. 2009). This picture differed in the case of *cnf* gene in the current study, and only 9.3% of strains were positive for this trait. Interestingly, this degree of consistency in the genetic background is in contrast to considerable serotype diversity observed in many investigations (Morabito *et al*. 2000; Schmidt *et al.* 2000; Kobayashi *et al*. 2009; Prager *et al*. 2009). The high prevalence of *astA* gene that encodes EAST1 (Enteroaggregative heat stable enterotoxin 1) should be particularly taken into consideration as the emerging highly pathogenic enteroaggregative haemorrhagic pathotype (EAHEC) harbour this virulence determinant (Brzuszkiewicz *et al*. 2011).

While there is insufficient information to consider *stx2f*-harbouring STEC as a normal inhabitant of the gastrointestinal tract of pigeons, the widespread occurrence of these organisms in Columbiformes (pigeons and doves) is a known fact. Morabito *et al.* (2001) recaptured 20 pigeons that initially were positive for

stx2f-STEC, but found that 15 were negative in secondary tests. Interestingly, the less developed cecum in pigeons does favour transient bacterial fauna (Baele *et al*. 2002; Silva *et al.* 2009). On the other hand, pigeons may acquire other STECs through diverse environmental sources. The experimental infection of pigeons by O157:H7 *E. coli* resulted in a carrier state for up to 29 days without any clinical signs (Cizek *et al*. 2000). These facts suggest pigeons gut to act as a possible niche for horizontal gene transfer and evolution of emerging pathogenic clones theoretically.

It is not clear at the moment that what selective forces mediate the maintenance of particular virulence determinants in the genome of strains carrying *stx2f* and preferential colonization in Columbiformes. Comparative genomic analysis is required to expand our knowledge about evolutionary perspective of these host adapted STEC strains with ability to infect humans.

Molecular fingerprinting and phylogenetic groups

Molecular fingerprinting by ERIC-PCR revealed 7 profiles (designated as A to G) in the 43 *stx2f*+ isolates (Figure 1). Profile A prevailed and was present in 65.1% (28/43) of isolates, and profile B was present in 23.25% of isolates. Only one isolate belonged to each of the remaining profiles (C-G), each with a frequency of 2.3%. In RAPD-PCR, 7 profiles were observed and designated as A to G (Figure 2). Profile A was present in 46.5% (20/43) of isolates; profile B was observed in 13.95%; E in 23.25%, and G in 9.3% of the isolates. Other profiles (C, D, F) each were observed with a frequency of 2.3% (Table 2). Combination of ERIC and RAPD-PCR discriminated 10 distinct profiles, which designated as A′ to J′ as follows: A′ (A+A), B′ (B+B), C′ (C+C), D′ (D+D), E′ (E+E), F′ (F+F), G′ (B+E), H′ (A+G), I′ (G+E), and J′ (A+E). The most prevalent profile was A′ with the frequency of 46.5% (20/43). The observed frequencies of A′ to J′ composite-profiles in different geographical areas are presented in Table 2.

Fingerprinting by the combination ERIC and RAPD-PCR increased the discriminatory power of methods in some instances. In RAPD-PCR, the discrimination of genotypes was similar to ERIC in two areas, but distribution of RAPD-types differed from ERIC in Tehran and Babol cities. As Table 2 shows, most of the genetic diversity was observed in isolates from Tehran and Garmsar prefectures. Clonal relatedness of *stx2f*+ *E. coli* isolated from pigeons was reported previously. In Italy pulse field gel electrophoresis (PFGE)

analysis showed that most of the strains from a flock exhibited similar profile; other isolates were related, but most of them revealed different patterns (Morabito *et al*. 2001).

In phylogenetic grouping, 40 strains (90%) only possessed the *TSPE4.C2* gene and were assigned to B1 phylogroup; the remaining 3 isolates harbored three genes and belonged to B2 phylogroup (Clermont et al., 2000). To our knowledge there is not any available information regarding phylogroups of *stx2f*+ strains at the moment, but the results of this study shows a high degree of similarity in phylogenetic groups of *stx2f*+ strains isolated from diverse geographical areas.

Serotyping

Serotyping of 10 selected representative strains showed that five strains belonged to O20 serogroup, four strains belonged to O78, and only one strain was positive for O115 somatic antigen. Seven strains out of ten were non-motile. A wide range of serogroups has also been previously reported in Stx2f-producing STECs worldwide including O15, O18, O20, O25, O45, O63, O75, O128, O132, O145, O152, O147, and O178 (Morabito *et al*. 2000; Schmidt *et al.* 2000; Kobayashi *et al*. 2009; Prager *et al*. 2009).

Sequencing of *stx2f* gene

Eight out of the ten examined strains produced the specific 625bp amplicon and were successfully sequenced. Two strains generated very faint specific bands that proved unsuitable for efficient sequencing. Interestingly, both of these strains showed the unique composite ERIC- RAPD profiles (C′ and D′). Multiple alignments by ClustalX of 500bp fragments from the eight sequences showed 100% identity. One of the obtained sequences was blasted in the nucleotide database deposited in the NCBI Gene Bank and 100% similarity was observed against most type strains of Stx2f-producing STEC including H.I.8 and O128: NM (accession numbers: AJ271139.1, AB499813.1). The sequence identity was estimated to be 99% against a few Stx2f-producing STEC (e.g. O115:NM: AB472687.1). The obtained sequence from T5B-Ir isolate (O115: NM) in this study was deposited in Gene Bank (KJ397538). According to the new standardized

nomenclature system for Shiga toxins (Scheutz *et al*. 2012), the current study did not show any variant for *stx2f* subtype in isolates from Iran. The predicted amino acid sequences of T5b-Ir strain in this study also aligned with some Stx2 subtypes in the reference protein database (Swiss-prot) using ClustalW as depicted in Figure S1.

Antibacterial susceptibility

All *stx2f*+ isolates were resistant to amoxicillin and lincospectin; while resistance to tetracycline, doxycycline and neomycin was observed in 88.4, 74.4 and 13.9% of the isolates, respectively. The most effective antibacterials were furazolidone, trimethoprim-sulfamethoxazole and gentamicin with the susceptibility rates of 95.3, 83.7, and 76.7% respectively. Noticeably, intermediate susceptibility to florfenicol, neomycin, and enrofloxacin was observed in majority of the isolates (Table 3). All strains showed multiple resistance, and most (81.4%) were resistant to four or more antibacterial agents.

There appears to be no previous report on the antibacterial susceptibility of *stx2f* possessing isolates. In the current study, resistance to some antibacterials was alarming as all isolates were consistently resistant to amoxicillin and lincospectin, and showed considerable resistance to tetracycline and doxycycline. Noticeable intermediate sensitivity to some potent antibacterials like neomycin, enrofloxacin, florfenicol, and ceftriaxone limits the choices of antimicrobial therapy as it has been suggested that strains with intermediate susceptibility to antibiotics should be considered resistant in clinical settings (Beco *et al*. 2013). Uncontrolled use of antibacterials by pigeon fancier can be considered an important factor contributing to high resistance against antibiotics. The spread of resistance genes via plasmids or other mobile genetic elements to other enteric bacteria means that domestic pigeons could be an important biological source for resistance.

Materials and Methods

Sampling, *E. coli* isolation and molecular characterization

A total number of 117 domestic pigeons were sampled from Tehran (n=25), Ferdows (n=31), Garmsar (n=28) and Babol (n=33) cities, which are located in Tehran, Khorasan, Semnan and Mazandaran provinces, respectively. The samples were collected from cloaca or fresh dropping of pigeons using sterile swabs and sent to the laboratory in Amies transport medium (BBL, USA) within 24h of collection. Samples were streaked on MacConkey agar, and up to four suspect pink colonies from each sample sub-cultured on LB agar. Crude bacterial DNA was extracted by the boiling method; then, strains were subjected to PCR using the specific primers for *stx2f* gene (Schmidt *et al*. 2000). Multiplex-PCR targeting *stx1*, *stx2*, *eae* and *ehly* was performed on all strains that possessed the *stx2f* gene (Paton and Paton 2002). The presence of additional virulence genes including *saa* (STEC autoagglutinating adhesin), *astA* (Enteroaggregative heat stable enterotoxin) and *cnf* (Cytotoxic necrotizing factor) was further investigated by PCR as described previously (Yamamoto *et al.* 1997; Paton and Paton 2002; Ewers *et al.* 2004). All *stx2f*-positive isolates were confirmed to be *Escherichia coli* using conventional biochemical tests and stored at -70°C in glycerol for further analysis.

Eleven strains containing *stx2f* and *eae* genes were randomly selected from different cities and subjected to a typing PCR for *eae* gene using one forward (EaeVF), and three reverse primers (Eae VR, Eae IotaVR, Eae ZetaVR). The 834 to 876bp specific products of the *eae* gene were subsequently subjected to restriction fragment length polymorphism (RFLP) and digested by *Alu*I , *Rsa*I and *Cfo*I (Fermentas, Lithuania) separately to determine the intimin subtypes according to Ramachandran *et al*. (2003).

Molecular fingerprinting

All isolates, which were positive for *stx2f* gene, were subjected to enterobacterial repetitive intergenic consensus sequences PCR (ERIC-PCR) and random amplified polymorphic DNA PCR (RAPD-PCR) fingerprinting methods. First, DNA was extracted from 18h LB broth culture; and concentration of DNA was

adjusted to 50ng/µl using a spectrophotometer (Sambrook *et al*. 2001). ERIC–PCR was performed with primers ERIC1 (5′-ATGTAAGCTCCTGGGGATTCAC-3′) and ERIC2 (5′-AAGTAAGTGACTGGGGTGAGCG-3′) as described by Versalovic *et al*. (1991). The PCR was conducted in a 25 µl volume containing 50 ng template DNA, 2 mmol l^{-1} MgCl2, 0.4 µmol l^{-1} primers, 1.25 U *Taq* DNA polymerase (Fermentas, Lithuania) and 200 µmol l^{-1} dNTP mix in 1xPCR buffer. The applied thermal cycles were as follow: initial denaturation at 94 °C for 7 min, 30 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 1 min, and extension at 72 °C for 3 min; followed by a final extension at 72 °C for 15 min. Electrophoresis was performed using 2% agarose gel at 70v for three hours.

In RAPD-PCR, primer 1247 (5′-AAGAGCCCGT-3′) was used (Heuvelink *et al*. 1995). The PCR was carried out in a 25 µl volume containing 50 ng template DNA, 2.5 mmol l^{-1} MgCl2, 0.4 µmol l^{-1} primer, 1.5 U *Taq* DNA polymerase (Fermentas, Lithuania) and 200 mmol l^{-1} dNTP mix in 1xPCR buffer. The PCR program was conducted as previously described by Schmidt *et al*. (1999), with the exception that electrophoresis applied for 4h at 70v. Both ERIC and RAPD-PCR were repeated twice to ensure reproducibility of results.

Phylogenetic analysis

The 43 *stx2f+ E. coli* isolates were subjected to phylogenetic group analysis according to Clermont et al. (2000). Strains were assigned to one of the four phylogenetic groups (A, B1, B2, D) based on the possession or absence of three genes (*chuA, TSPE4.C2, yjaA*). Multiplex-PCR conducted in 25 µl using 50 ng template DNA, 1.5 mM MgCl2, 0.5 µM of each primers, 1U *Taq* DNA polymerase (Cinnagen, Iran) and 200 µM dNTP mix in 1xPCR buffer. The applied thermal cycles were consisted of 94°C for 35s, 59°C for 15s, and 72°C for 20s. The annealing temperature in the initial 5 cycles was 61°C for 30s, decreasing to 60 in the next 5 cycles, and 59°C in the remaining 23 cycles.

Serotyping

A total number of ten representative strains showing different composite-profiles were chosen with respect to different geographical areas and subjected to serogrouping of O-antigens using commercial antisera (Mast diagnostics, UK) according to the manufacturer's instruction. Motility of these strains was also tested by inoculation into motility test medium to demonstrate the presence of flagellar antigens.

Sequencing of *stx2f* gene

Ten strains with different composite-profiles (the same strains as described above) were subjected to amplification of the *stx2f* gene using f4-f and R-e/f primers as described for sequencing of *stx2f* gene previously (Scheutz *et al.* 2012). The quality of PCR products was verified by electerophoresis in agarose gels, and the suitable products with sharp specific bands were sequenced using ABI 3731xl (Applied Biosystems) automated sequencer.

Antibacterial Susceptibility

Antibacterial Susceptibility of the *stx2f*+ strains was determined on Mueller-Hinton agar (Merk, Germany) by Kirby-Bauer method according to CLSI protocol (CLSI 2008). Twelve antibacterial discs were used including amoxicillin, gentamicin, neomycin, enrofloxacin, lincospectin, doxycycline, tetracycline, furazolidon, trimethoprim, trimethoprim-sulfamethoxazole, florfenicol and ceftriaxone.

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Conflict of Interest

No conflict of interest declared.

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Supporting Information

Figure S1. Multiple alignments of the predicted amino acid sequences of the strain T5b-Ir in this study (KJ397538) with some reference protein database for Stx2. Partial (79 amino acid) fragment is shown.

Table 1. Prevalence of *stx2f*+ isolates in pigeons from different geographical areas

Geographic area (City)	Samples No.	*stx2f*+ samples	*stx2f*+ isolates	Frequency (%)†
Tehran	25	5	10	20
Ferdows	31	8	8	25.8
Garmsar	28	6	18	21.4
Babol	33	3	7	9.09
total	117	22	43	18.8

† Percentage of positive samples

Table 2. Distribution of fingerprinting patterns of 43 *stx2f*+ *E. coli* isolates in different geographical areas

Geographical Area	Tehran	Ferdows	Garmsar	Babol
Fingerprinting	**Profile: n (%)**			
ERIC-Types	**A:** 9 (20.9) **G:** 1 (2.3)	**A:** 6 (13.9) **C:** 1 (2.3) **D:** 1 (2.3)	**A:** 12 (27.9) **B:** 6 (13.9)	**A:** 1 (2.3) **B:** 4 (9.3) **E:** 1 (2.3) **F:** 1 (2.3)
RAPD-Types	**A:** 1 (2.3) **E:** 5 (11.6) **G:** 4 (9.3)	**A:** 6 (13.9) **C:** 1 (2.3) **D:** 1 (2.3)	**A:** 12 (27.9) **B:** 6 (13.9)	**A:** 1 (2.3) **E:** 5 (11.6) **F:** 1 (2.3)
ERIC+RAPD-Types	**A′:** 1 (2.3) **H′:** 4 (9.3) **I′:** 1 (2.3) **J′:** 4 (9.3)	**A′:** 6 (13.9) **C′:** 1 (2.3) **D′:** 1 (2.3)	**A′:** 12 (27.9) **B′:** 6 (13.9)	**A′:** 1 (2.3) **E′:** 1 (2.3) **F′:** 1 (2.3) **G′:** 4 (9.3)

Table 3. Antibacterial susceptibility of 43 *stx2f*+ isolates in this study

Antibacterials	Resistant	Intermediate	Susceptible
		n (%)	
Neomycin	6 (13.95)	37 (86.04)	0
Trimethoprim	0	18 (41.86)	25 (58.13)
Furazolidone	0	2 (4.65)	41 (95.34)
Ceftriaxone	0	21(48.83)	22 (51.16)
Enrofloxacin	0	34 (79.06)	9 (20.93)
Tetracycline	38 (88.37)	4 (9.30)	1 (2.32)
Lincospectin	43 (100)	0	0
Doxycycline	32 (74.41)	9 (20.93)	2 (4.65)
Trimethoprim-sulfa	0	7 (16.27)	36(83.72)
Amoxicillin	43 (100)	0	0
Gentamicin	0	10 (23.25)	33 (76.74)
Florfenicol	0	37 (86.04)	6 (13.95)