Pathotyping of diarrhoeagenic cattle Escherichia coli *strains isolated in the Province of Tehran, Iran*

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Summary
An oligonucleotide DNA microarray targeting 348 virulence factors and genetic markers was used in the pathotyping, serotyping and phylogrouping of 51 *Escherichia coli* strains isolated from faecal samples. The samples were collected from diarrhoeic 1 to 30 days old calves located at 14 farms in the Tehran province, Iran. Positive microarray signals for genes encoding the Locus of Enterocyte Effacement (LEE), the Type III Secretion System (TTSS), and the absence of EPEC adherence factor (EAF) permitted the pathotyping of 25 strains as atypical Enteropathogenic (aEPEC) or Enterohaemorrhagic *Escherichia coli* (EHEC). The lack of LEE and TTSS-associated genes distinguished the remaining 26 strains, which were classified as Extraintestinal pathogenic *E. coli* (ExPEC). Atypical EPEC belonged to phylogroup B1 and possessed a LEE profile *tir-1, eae(beta), espA-1, espB-3*. The EHEC strains primarily belonged to the B1 phylogroup type-O26 and possessed either a LEE profile *tir-1, eae(beta), espA-1, espB-3*, or a B1 type-O111, LEE *tir-3, eae(gamma), espA-1, espB-2*. ExPEC-typed strains generally harboured genes localised to the constant region of Colicin V plasmid (pColV), including increased serum survival factor (*iss*), complement resistance protein (*traT*), aerobactin operon (*iucD*), and the siderophore receptor (*iroN*). The microarray platform used in this study is well suited to accurately and rapidly type attaching and effacing *E. coli* (AEEC-types), thus providing a database for the meta-analysis of ExPEC-typed strains.

Patotipi di Escherichia coli *isolati da vitelli diarroici della Provincia di Teheran, Iran*

Parole chiave
Bovino,
Escherichia coli,
ExPEC,
Iran,
InPEC,
Microarray.

Riassunto
Nel presente lavoro è stato utilizzato un DNA microarray disegnato con sonde oligonucleotiche nei confronti di 348 fattori di virulenza e marker genetici per determinare l'appartenenza al patotipo, al sierotipo e al filogruppo di 51 ceppi di *Escherichia coli* isolati da campioni di feci. I campioni sono stati raccolti da vitelli con diarrea, di età compresa tra 1 e 30 giorni, provenienti da 14 allevamenti situati nella provincia di Teheran, Iran. La positività dell'ibridizzazione nei confronti di geni codificanti il Locus di Enterocyte Effacement (LEE) e il Type III Secretion System (TTSS), e l'assenza di segnale nei confronti del fattore di aderenza di *E. coli* (EAF), ha permesso di determinare l'appartenenza di 25 ceppi al patotipo aEPEC (atypical Enteropathogenic *E. coli*) o EHEC (Enterohaemorrhagic *E. coli*). L'assenza di segnale nei confronti di geni del LEE e di geni TTSS-associati hanno caratterizzato i restanti 26 ceppi, classificati come ExPEC (Extraintestinal Pathogenic *E. coli*). I ceppi aEPEC possedevano i marker genetici del filogruppo B1 e il loro profilo LEE è risultato caratterizzato dalle seguenti varianti genetiche: *tir-1, eae(beta), espA-1, espB-3*. I ceppi EHEC sono risultati prevalentemente classificati nel filogruppo B1 e il loro profilo LEE è risultato caratterizzato dalle varianti *tir-1, eae(beta), espA-1, espB-3*, in sierotipi O26, o dalle varianti *tir-3, eae(gamma), espA-1, espB-2*, in sierotipi O111. I ceppi classificati come ExPEC in generale ospitavano geni localizzati nella regione costante del plasmide Colicin V (pColV), comprendenti i geni *iss* (increased serum survival factor), *traT* (complement resistance protein), *iucD* (aerobactin operon) e *iroN* (siderophore receptor). La tecnologia microarray utilizzata in questo studio si è dimostrata

adatta nel tipizzare con precisione e facilità ceppi di *E. coli* appartenenti ai patotipi AEEC (Attaching and Effacing *E. coli*). Inoltre essa ha fornito un database genetico utile alla meta-analisi di ceppi ExPEC.

Introduction

Pathogenic *Escherichia coli* (PEC) has evolved by acquiring virulence factors through transfer of foreign DNA mediated by mobile genetic elements, such as conjugative plasmids, transposons, bacteriophages, pathogenicity islands (PAI), as well as DNA recombination. Based on histological characters and target organs, where they exert their pathogenic effects, PECs can be grouped into intestinal and extraintestinal types, also called InPEC and ExPEC (Schmidt and Hensel 2004, Johnson and Nolan 2009).

Due to their particular virulence gene content and overt diarrhoeic symptoms, InPECs are classified as enteropathogenic (EPEC), enterohemorrhagic (EHEC), enterotoxigenic (ETEC), enteroaggregative (EAEC), enteroinvasive (EIEC), and diffusely adherent (DAEC) pathotypes. The EPEC and EHEC pathotypes possess a chromosomal PAI named Locus of Enterocyte Effacement (LEE), encoding adhesion factors (Ler, Eae, Tir), structural, and effector proteins (Esc, Esp, Epr) of type III secretion system (TTSS), which enable the bacterium to adhere to intestinal epithelial cells and cause attaching-and-effacing (A/E) histopathologic lesions (Kaper *et al.* 2004). Furthermore, A/E strains encode non-LEE TTSS-translocated effectors (*cif, map, nle*), colonization (*paa, efa1/lifA, lpfA*) and plasmid virulence factors (*ureD, ehx, iha, espP, katP*) (Batisson *et al.* 2003, Marches *et al.* 2003, Marches *et al.* 2005). Enteropathogenic strains have been subdivided into typical and atypical (tEPEC and aEPEC), with the strains differing from each other in the colonization of intestinal epithelial cells. The adherence of tEPEC strains is mediated by bundle-forming pili (*bfp* gene cluster) encoded by an EPEC adherence factor (EAF)-type plasmid. The aEPEC do not carrying EAF plasmids and are responsible for prolonged, but not bloody, diarrhoeic syndrome in children. They share similarities with EHEC and *bfp*-negative enteropathogenic *E. coli* strains from environmental or animal origin (Nguyen *et al.* 2006).

Differences between the EPEC and EHEC groups are based on the absence or presence of genes encoding Shiga toxins (*stx1AB* and/or *stx2*). The enterohemorrhagic strains or other strains capable of producing Shiga toxins, regardless of whether they possess or not the LEE or TTSS genes, are also named Shiga toxin *E. coli* (STEC). They are associated with bloody diarrhoea in humans. The EHEC-associated bloody diarrhoea is a risk factor for the development of a separate syndrome defined by haemolytic anaemia, thrombocytopenia, and renal failure (Bugarel *et al.* 2010). Although the various EHEC serotype members of O26, O91, O103, O104, O111, O113, O145, non-motile O157, and some other serotypes are frequently associated with human disease, *E. coli* O157:H7 is the most important member of this group. Ruminants, particularly cattle, are the most important reservoirs for EHEC O157:H7 and play a role in the spread of the infection to human beings and other animals (Sheng *et al.* 2006). Unlike O157, non-O157 EHEC serotypes can induce diarrhoea in young ruminant animals (Tòth *et al.* 2009).

Other diarrhoeagenic *E. coli* strains include ETECs, which adheres to the small intestine via F type fimbriae and colonization factors. The ETEC toxins are plasmid-encoded heat-labile (LT) and/or heat-stable (ST) enterotoxins and can cause human, porcine, and bovine diarrhoea. The EAEC pathogenesis involves adherence of bacteria to intestinal mucosa through aggregative adherent fimbriae (AAF) in a 'stacked-brick' configuration producing a mucous-mediated biofilm on the enterocyte surface. While EAEC-released toxins affect the inflammatory response, intestinal secretion, and mucosal cytotoxicity. Diffusely adherent strains are neither invasive nor toxigenic, but are defined on the basis of diffuse adherence on cultured cells. The EIEC is a non-toxigenic diarrheagenic pathotype, closely related to *Shigella* spp., and is characterized for its ability to provoke invasive intestinal infections in humans and animals. (Kaper *et al.* 2004, Okeke and Nataro 2001, Johnson and Nolan 2009, Fleckenstein *et al.* 2010).

In contrast to InPECs, there is little agreement on the genetic determinants defining ExPECs. Since these strains normally colonize the gut of healthy hosts, like commensal *E. coli*, this poses the question as to whether ExPECs are essentially opportunistic pathogens. The ExPEC pathotypes lack LEE-encoding genes as well as plasmid or chromosomal-bearing colonization factors and effectors normally involved in InPEC pathogenesis. Nonetheless, they have been classified into uropathogenic (UPEC), neonatal-meningitis associated (MNEC), and septicemic *E. coli* (SEPEC) pathotypes (Russo and Johnson 2000, Köhler and Dobrindt 2011). The

ExPEC-associated virulence factors include fimbrial and pilum adhesins-encoding genes (F/S fimbriae, *sfa*, *foc*; pyelonephritis-associated pili, *pap*; Dr family adhesins, *afa*, *dra*), invasins (*ibeAB*), toxins (cytolethal distending toxin, *cdt*; cytotoxic necrotizing factor, *cnf*; hemolysin, *hly*), surface antigens (capsule syntesis, *KpsM*, *neuAC*; enterobactin receptor, *iha*), iron uptake (*chuA, fepC, irp, fyuA, iroN, iucD*), and ColV plasmid-encoded PAI (aerobactin receptor, *iutA*; iron transport system, *sitABCD*; outer membrane, *ompT*; increased serum survival, *iss*; temperature-sensitive hemolysin, *tsh*; and avian hemolysin, *hlyF*).

Although some gene patterns have been proposed to define the UPEC (*papA/C/G, hlyA, cnf1*, iron acquisition and specific PAIs), SEPEC (*cdtB, cnf, F17A, f165-1A, gafD, iucD*), and MNEC (*ibeA, neuA, neuC*) pathotypes, any classification scheme should assume that a common set of virulence factors is undoubtedly required to generate a symptomatic response (Hill 2013). A possible key to understand the virulence gene composition of ExPECs comes from studies using sequenced Avian Pathogenic *E. coli* (APEC) genomes. Avian colibacillosis predominantly occurs in extra intestinal sites, inducing septicemia, airsacculitis-polyserositis, yolk sac infection, and salpingitis, cellulitis, resulting in acute, sub-acute and chronic diseases. The APEC genomes show remarkable similarities with the ExPEC genomes, especially UPEC strains possessing the *pap* fimbrial operon, yersiniabactin siderophore gene (*fyuA*), salmochelin siderophore gene (*iroN*), iron transport gene (*sitABCD*), and the aforementioned ColV plasmid-encoded PAI (Rodriguez-Siek *et al.* 2005, Johnson *et al.* 2007, Johnson and Nolan 2009, Kabir, 2010).

A separate classification scheme for pathogenic *E. coli* involves the clustering of strains into 4 phylogenetic groups based on the presence/absence of the heme transport gene *chuA*, the genetic marker *yjaA*, and the putative lipase esterase DNA fragment TspE4.C2, as proposed by Clermont and colleagues (Clermont *et al.* 2000). Groups A and B1 are primarily composed of commensal, low-pathogenic or enteropathogenic *E. coli* mainly associated with animals, while group B2 and, to a lesser extent, group D includes the majority of virulent human extra-intestinal *E. coli* (Carlos *et al.* 2010).

In Iran, the relatively few epidemiological studies and the absence of a surveillance system for diarrhoeagenic *E. coli* limit our understanding of the impact of pathogenic *E. coli* on animal and human health in this country (Jafari *et al.* 2012). However, data from the Iranian Nosocomial Infection Surveillance System demonstrate a high instance of urinary tract and bloodstream infections in children resulting in hospitalization (Zahraei *et al.* 2012). Furthermore, some recent investigations about diarrhoeagenic *E. coli* have focused on hospitalized human patients in Tehran and other areas of the country (Jafari *et al.* 2009, Bonyadian *et al.* 2010), diarrhoeic and healthy calves in Tehran (Badouei *et al.* 2010), and slaughtered sheep in Shiraz (Tahamtan *et al.* 2010). In these studies, non-O157 Shiga toxin-harbouring *E. coli* was prevalent. In contrast, *E. coli* O157:H7 was found in stool specimens of children (Salmanzadeh-Ahrabi *et al.* 2005), cattle carcasses, diarrhoeic calves (Hashemi *et al.* 2010, Shahrani *et al.* 2014), raw milk from ruminants (Rahimi *et al.* 2012), and camel faeces (Salehi *et al.* 2012). Finally, some recent studies on *E. coli* isolated from healthy and/or diseased poultry and classified as APEC showed virulence gene patterns composed of *iss-hlyF-ompT-iroN* and *iss-tsh* genes (Arabi *et al.* 2013, Kafshdouzan *et al.* 2013).

An oligonucleotide microarray, initially developed and validated as previously described (Bekal *et al.* 2003, Bruant *et al.* 2006, Jakobsen *et al.* 2011), allows for the detection of an exhaustive list of *E. coli* virulence genes as well as gram-negative antimicrobial resistance genes. The array, or its upgraded version, has been useful in assessing the virulence gene composition of *E. coli* isolated from coastal and waste waters (Frigon *et al.* 2013, Hill 2013), camels (Salehi *et al.* 2012), poultry (Bonnet *et al.* 2009), and rabbits (Badagliacca *et al.* 2016).

The present study expands our knowledge on the circulation of pathogen-associated virulence genes in the most populated area of Iran through the molecular typing of *E. coli* isolates by DNA microarray from outbreaks of calf colibacillosis.

Materials and methods

Strains, isolation, and DNA extraction

A collection of 51 *E. coli* strains was isolated from faecal samples collected from diarrhoeic 1 to 30 days old calves from 14 farms located in the Tehran province, Iran (Figure 1). *Escherichia coli* isolation was performed according to the protocol described by Alonso and colleagues (Alonso *et al.* 1999). Briefly, faecal samples were cultured on CHROMagarECC (CHROMagar Microbiology, Paris, France) and incubated at 41°C. All β-galactosidase (LAC) and β-glucuronidase positive isolates (blue colonies) were selected. Biochemically confirmation was obtained via commercial miniaturized kit. Confirmed samples were considered to be *E. coli*. DNA was extracted using Maxwell 16 Cell DNA Purification Kit (Promega, Milano, Italy) according to manufacturer's protocol, and stored at -20°C until use.

Microarray design

The microarray version used in the present study was

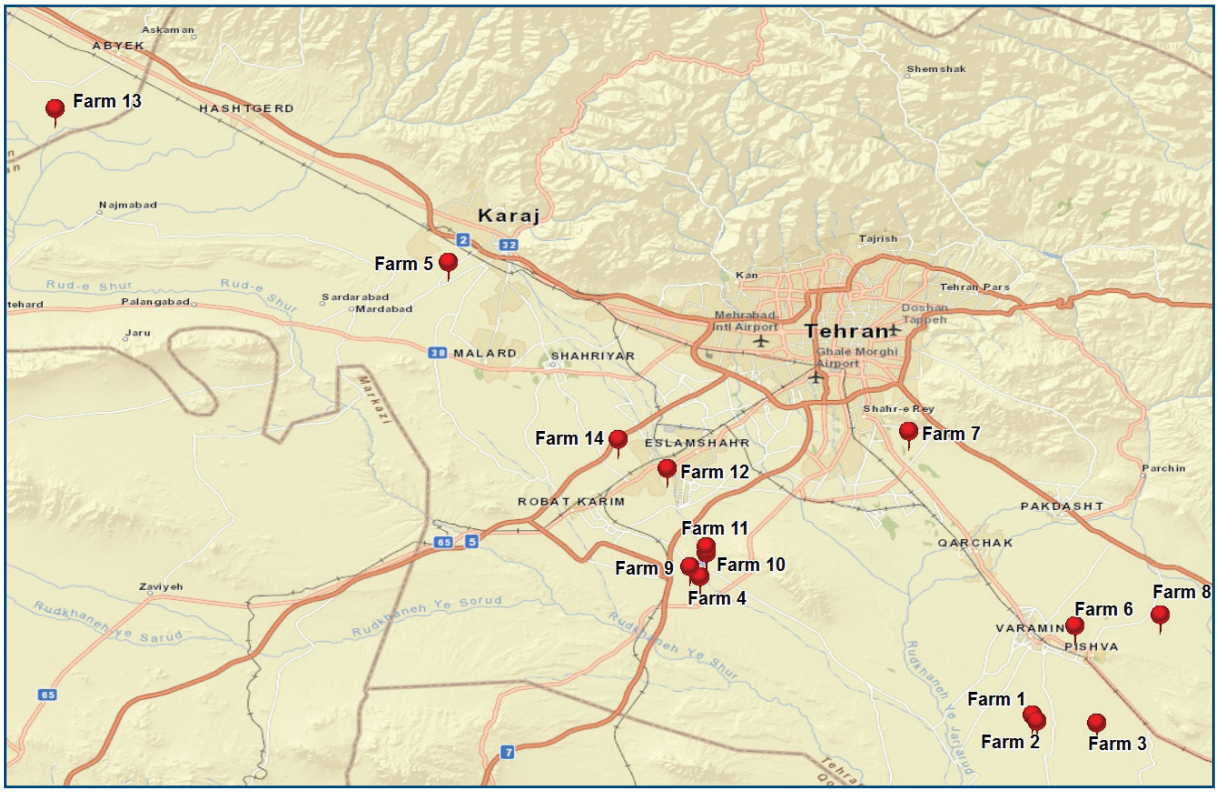

Figure 1. *Location of farms and number of* E.coli *strains isolated.* Farms 1 to 3 were located in Javad abad and 4, 3 and 5 strains were isolated from them, respectively; farms 4 and 9 in Hasan abad (3 and 2 strains); farm 5 in Mard abad (1 strain); farms 6 and 8 in Varamin (2 and 4 strains); farm 7 in Aminabad (5 strains); farms 10 and 11 in Fashafuyeh (6 and 5 strains); farms 12 and 14 in Eslam shahr/Saveh road (6 and 2 strains); farm 13 in Abyek ahmad abad (3 strains).

composed of 70-mer oligonucleotide probes printed in duplicate on Corning Ultra GAPS slides (Corning Canada, Whitby, Ontario, Canada), targeting 348 *E. coli* virulence factors, and covering all known *E. coli* pathotypes, including 95 antimicrobial resistance genes. The tryptophanase (*tnaA*), beta-glucuronidase (*uidA*), lactose permease (*lacY*), and beta-galactosidase (*lacZ*) genes were included as positive controls; whilst negative controls included empty buffer spots as well as genes for the green fluorescent protein of *Aequoria victoria* (*gfp*) and the chlorophyll synthase gene of *Arabidopsis thaliana* (*At3g*). Only data related to virulence or virulence-related gene hybridizations were considered in this study, since the antimicrobial resistance profiles of the same strains have been analysed separately (Badagliacca *et al.* 2014).

DNA labeling, hybridization and microarray data acquisition

The purified genomic DNA was quantified using a Nanodrop Spectrophotometer (Nanodrop Technologies, Celbio Srl., Milan, Italy), labelled and submitted to microarray hybridization as previously described (Badagliacca *et al.* 2014, Badagliacca *et al.* 2016). Microarray slides were scanned with a ScanArray Lite fluorescent microarray analysis system (Perkin-Elmer, Mississauga, Ontario, Canada) using ScanArray Gx software (Perkin-Elmer, Foster City, CA). Fluorescent spot intensities were quantified with QuantArray Version 3.0 (Packard Bioscience). 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 (2 in this study) were considered positive. These ratios were then converted into binary data, where 0 indicates a negative probe and 1 indicates a positive probe.

Pathotype, serogroup, and phylogroup assignment rules

According to the relevant literature (Rodriguez-Siek *et al.* 2005, Hamelin *et al.* 2006, Johnson *et al.* 2008, Johnson and Nolan 2009, Kohler and Dobrindt 2011, Schouler *et al.* 2012, Hill 2013), assignment to attaching and effacing *E. coli* (AEEC) type was based on possessing the LEE functional domain-encoding genes (*ler, tir, eae, espA, espB*) and LEE or non-LEE effector protein-encoding genes (*map, espG, nle* family genes). To subdivide further AEEC-specific

pathotypes, tEPEC or aEPEC strains either possessed or lacked the bundle forming pilus *bfp* family genes, respectively. Strains possessing Shiga toxin-encoding *stx1* and/or *stx2* family genes were classified as EHEC. The AEEC-negative InPEC strains were identified on the basis of the presence of colonization factors and toxin-encoding genes related to ETEC (coli surface antigen CS family genes, K88 F4 fimbrial-encoding gene, heat-stable enterotoxins, heat-labile enterotoxins), to EAEC, DAEC (genetic markers *agg, aaf, aap* for pAA plasmid, genetic marker *virK, capU, shf* for pAA2 plasmid and *daa* Dr family adhesins, respectively), and to EIEC (marker for the invasion plasmid antigen, *ipaH*). Since characterizing ExPECs strains into specific pathotypes could be ambiguous, assignment was done on the basis of prevalent patterns related to positive signals for fimbrial factors (*pap* family genes, F/S fimbriae), toxins (*hly, hra, cnf, senB*), adhesins, and invasins (*afa, nfa, tib, ipaH*), iron acquisition (*chuA, fepC, iroN, fyuA, iut, sit, irp*), capsule synthesis (*kpsM, neuAC*), and pColV plasmid determinants (*iss, traT, ompT, tsh*, and colicin structural genes).

Escherichia coli isolates were assigned to a serogroup based on their *wzy* gene for O7, O15, O22, O24, O26, O28, O45, O53, O55, O56, O59, O66, O86, O91, O98, O103, O104, O113, O114, O117, O121, O123, O126, O127, O128, O138, O139, O141, O145, O146, O147, O148, O149, O155, O157, O172, O174, and O177. Assignment to other serogroups was based on the *rfc* gene for O4, *wzx* gene for O6, *wb* for O8, *rfb* for the O9, and O101 and *wbdI* gene for O111. *Escherichia coli* phylogenetic group assignment was based on the presence/absence of *chuA, yjaA*, and TspE4.C2 according to Clermont and colleagues (Clermont *et al.* 2000).

Results

Strain screening using a complete genetic set of the LEE functional domains, the positive signals for genes encoding structural proteins of TTSS, the LEE and the non-LEE effector proteins involved in enteritis pathogenesis, and the absence of EAF plasmid determinants showed that 25 of 51 isolates were AEEC or more specifically, 4 aEPEC, and 21 EHEC/STEC pathotypes. The absence of complete LEE and TTSS-associated genes distinguished the remaining 26 strains. These strains showed genetic patterns related to the presence of fimbrial factors, toxins, adhesins, iron uptake, capsule synthesis, and colicin plasmid markers, thus classifying them as ExPEC strains. Among the ExPECs, a cluster of 13 primarily UPEC/APEC strains was differentiated from 2 other groups of APEC and undefined ExPEC strains, respectively.

Genetic profiles of InPEC strains

Table I and II summarize the genetic characterization of aEPEC and EHEC found in the AEEC cluster, respectively. The 4 aEPEC strains all belonged to phylogroup B1 and showed a common genetic core consisting of a LEE profile (*tir-1, eae(beta), espA-1, espB-3* variants), the adhesion factor long polar fimbriae (*lpfA*), the lymphocyte activation inhibitor (*lifA*), and the TTSS-translocated effectors *espG, map* and/or *cif*, attesting to their ability to induce intestinal lesions. No correlations were found between the genetic profile and serogroup or farm of origin.

The EHEC-typed isolates, including both LEE and Shiga toxin-harbouring *E. coli* strains, were primarily from phylogroup B1, with the exception of 2 strains belonging to the B2 and 4 strains belonging to the D phylogroups. These strains all possessed the A and B subunits of *stx1*, and 6 of them were also positive to the A and/or subunits B of *stx2*. They also showed 1 or more pO157 plasmid markers (*katP, ehxA, espP, iha, ureD*). Their genetic set of colonization factors included the above mentioned pattern *lpfA-lifA*, in addition to the porcine A/E associated protein, *paa*. The LEE profiles concerning the prevalent B1-phylogroup O26 (7 strains) and O111 (8 strains) serogroup were *tir-1, eae(beta), espA-1, espB-3, and tir-3, eae(gamma), espA-1, espB-2*, respectively. The B2 or D-phylogroup O157 (2 strains), the D-phylogroup O155 (1 strains),

Table I. *Prevalent virulence factors characterizing atypical EPEC strains.*

Strain*	Sero-type	Phylo-type	LEE functional domain profile	LEE and non-LEE effector	Colonization factors and adhesin	Plasmid virulence gene
2-C	O123	B1	*ler, tir-1, eae, eae(beta), espA-1, espB-3, escN, eprJ*	*cif, espG, map(1), nleA, nleB, nleC, nleE, nleF, nleG, nleH*	*paa, efa1(lifA), lpfA*	*ureD, set*
10-D	NT	B1	*ler, tir-1, eae, eae(beta), espA-1, espB-3, escN, eprJ*	*espG, map, nleA, nleB, nleE, nleF, nleG, nleH*	*efa1(lifA), lpfA*	*ehxA, iha, ureD*
11-E	NT	B1	*ler, tir-1, eae, eae(beta), espA-1, espB-3, escN, eprJ*	*cif, espG, map, nleA, nleB, nleC, nleE, nleF, nleG, nleH*	*efa1(lifA), lpfA,*	*ehxA, iha, ureD, set*
14-B	O8	B1	*ler, tir-1, eae, eae(beta), espA-1, espB-3, escN, escJ, eprJ*	*cif, espG, map, nleA, nleB, nleC, nleD, nleE, nleF, nleG, nleH*	*paa, efa1(lifA), lpfA*	*ehxA, iha, ureD, set, ECs1282, rtx*

*Strain identification was coded by Farm number (from Figure 1) and letter (strain isolate); NT = nontypeable.

Table II. *Prevalent virulence factors characterizing EHEC strains.*

Strain*	Sero-type	Phylo-type	LEE functional domain profile	Shiga toxin	LEE and non-LEE effector	Colonization factors and adhesin	Plasmid virulence gene
2-A	O157	B2	*ler, tir-2, eae, eae(gamma2), espA-2, espB-1; escJ, escN, eprJ*	*stx1A, stx1B, stx2A, stx2B-1*	*map(3), nleA, nleB, nleC, nleD, nleE, nleF, nleG, nleH, set, rtx, ECs1282, tccP*	*paa, efa1(lifA), lpfA, afaD*	*espP, ehxA, katP, etpD, L7095, iha, rfbE, ureD*
2-B	O26	B1	*ler, tir-1, eae, eae(beta), espA-1, espB-3, escN, eprJ*	*stx1A, stx1B*	*cif, espG, map(1), nleA, nleB, nleC, nleE, nleF, nleG, nleH*	*paa, efa1(lifA), lpfA*	*iha, ureD, set*
3-A	O26	B1	*ler, tir-1, eae, eae(beta), espA-1, espB-3, escJ, escN, eprJ*	*stx1A, stx1B*	*cif, espG, map(1), nleA, nleB, nleC, nleD, nleE, nleF, nleG, nleH*	*paa, efa1(lifA), lpfA,*	*espP, ehxA, iha, ureD, set*
3-B	O26	B1	*ler, tir-1, eae, eae(beta), espA-1, espB-3, escN, eprJ*	*stx1A, stx1B, stx2B*	*cif, espG, map(1), nleA, nleB, nleC, nleE, nleF, nleG, nleH*	*paa, efa1(lifA), lpfA*	*espP, ehxA, katP, iha, ureD, set*
3-D	O111	B1	*ler, tir-3, eae, eae(gamma), espA-1, espB-2, escN, escJ, eprJ*	*stx1A, stx1B, stx2A*	*cif, espG, nleA, nleB, nleC, nleE, nleG, nleH*	*paa, efa1(lifA), lpfA*	*ehxA, iha, ureD, set*
3-E	O26	B1	*ler, tir-1, eae, eae(beta), espA-1, espB-3, escN, escJ, eprJ*	*stx1A, stx1B*	*cif, espG, map, nleA, nleB, nleC, nleE, nleF, nleG, nleH*	*paa, efa1(lifA), lpfA*	*iha, ureD, set*
4-A	NT	D	*eae, eae(gamma2), espA-2, espB-1, escJ, eprJ*	*stx1A, stx1B*	*map, nleD, nleE*	*paa, lpfA,*	*espP, etpD, iha, rfbE, ureD, set*
5-A	O26	B1	*ler, tir-1, eae, eae(beta), espA-1, espB-3, escN, eprJ*	*stx1A, stx1B*	*cif, espG, map, nleA, nleB, nleC, nleE, nleF, nleG, nleH*	*paa, efa1(lifA), lpfA,*	*iha, ureD, set*
6-B	O26	B1	*ler, tir-1, eae, eae(beta), espA-1, espB-3, escN, escJ, eprJ*	*stx1A, stx1B*	*cif, espG, map, nleA, nleB, nleC, nleE, nleG, nleH*	*efa1(lifA), lpfA,*	*espP, ehxA, katP, iha, ureD, set*
7-A	O155	D	*ler, tir-3, eae, eae(gamma2), espA-2, espB-1, escN, escJ, eprJ*	*stx1A, stx1B, stx2B*	*espG, map, nleA, nleB, nleC, nleD, nleE, nleF, nleG, nleH*	*paa, efa1(lifA), lpfA,*	*espP, ehxA, iha, rfbE, ureD, katP, rtx*
7-B	O111	B1	*ler, tir-3, eae, eae(gamma), espA-1, espB-2, escN, escJ, eprJ*	*stx1A, stx1B*	*cif, espG, map, nleA, nleB, nleC, nleE, nleG, nleH*	*paa, efa1(lifA), lpfA,*	*iha, ureD, set*
7-C	O157	D	*ler, tir-3, eae, eae(gamma2), espA-2, espB-1, escN, escJ, eprJ*	*stx1A, stx1B*	*espG, map, nleA, nleB, nleC, nleD, nleE, nleF, nleG, nleH, tccP,*	*paa, efa1(lifA), lpfA,*	*espP, ehxA, katP, etpD, iha, rfbE, ureD, set, rtx, L7095*
7-D	NT	D	*ler, tir-3, eae, eae(gamma2), espA-2, espB-1, escN, escJ, eprJ*	*stx1A, stx1B*	*espG, map, nleA, nleB, nleC, nleD, nleE, nleF, nleG, nleH,*	*lpfA*	*espP, ehxA, katP, etpD, iha, ureD, set, rtx*
8-A	O111	B1	*ler, tir-3, eae, eae(gamma), espA-1, espB-2, escN, escJ, eprJ*	*stx1A, stx1B*	*cif, espG, map, nleA, nleB, nleC, nleE, nleG, nleH*	*paa, efa1(lifA), lpfA*	*ehxA, iha, ureD, set*
8-B	O26	B2	*ler, tir-1, eae, eae(beta), espA-1, espB-3, escN, escJ, eprJ*	*stx1A, stx1B, stx2B*	*cif, espG, map, nleA, nleB, nleC, nleD, nleE, nleF, nleG, nleH, tccP*	*paa, efa1(lifA), lpfA*	*espP, rfbE, ehxA, katP, iha, ureD, set, rtx, L7095*
8-C	O111	B1	*ler, tir-3, eae, eae(gamma), espA-1, espB-2, escN, escJ, eprJ*	*stx1A, stx1B*	*cif, espG, map, nleA, nleB, nleC, nleE, nleG, nleH*	*paa, efa1(lifA), lpfA*	*ehxA, iha, ureD, set*
8-D	O26	B1	*ler, tir-1, eae, eae(beta), espA-1, espB-3, escN, eprJ*	*stx1A, stx1B*	*cif, espG, map, nleA, nleB, nleC, nleE, nleF, nleG, nleH*	*paa, efa1(lifA), lpfA*	*espP, ehxA, katP, iha, ureD, set*
12-A	O111	B1	*ler, tir-3, eae, eae(gamma), espA-1, espB-2, , escN, escJ, eprJ*	*stx1A, stx1B, stx2B*	*cif, espG, map, nleA, nleB, nleC, nleE, nleG, nleH*	*paa, efa1(lifA), lpfA,*	*ehxA, iha, ureD, set*
12-B	O111	B1	*ler, tir-3, eae, eae(gamma), espA-1, espB-2, escN, escJ, eprJ*	*stx1A, stx1B*	*cif, espG, map, nleA, nleB, nleC, nleE, nleG, nleH*	*paa, efa1(lifA), lpfA*	*ehxA, iha, ureD, set*
12-C	O111	B1	*ler, tir-3, eae, eae(gamma), espA-1, espB-2, escN, escJ, eprJ*	*stx1A, stx1B*	*cif, espG, map, nleA, nleB, nleC, nleE, nleG, nleH,*	*paa, efa1(lifA), lpfA*	*ehxA, iha, ureD, set, ECs1282, L7095, rtx*
13-C	O111	B1	*ler, tir-3, eae, eae(gamma), espA-1, espB-2, escN, escJ, eprJ*	*stx1A, stx1B*	*cif, espG, map, nleA, nleB, nleC, nleE, nleG, nleH*	*paa, efa1(lifA), lpfA*	*ehxA, iha, ureD, set*

* Strain identification was coded by Farm number (from Figure 1) and letter (strain isolate); NT = nontypeable.

and the D-phylogroup O-antigen nontypeable (2 strains) possessed a similar LEE profile, *tir-2* or *tir-3, eae(gamma2), espA-2, espB-1*. Furthermore, LEE- and non-LEE-TTSS effectors overlapping the above mentioned pattern for aEPEC strains, were also detected in the EHEC group.

Genetic profiles of ExPEC strains

In order to pathotype the 26 ExPEC strains found in our *E. coli* collection, Tables III to VI show the genetic profiles related to fimbrial factors, toxins, adhesins, and invasins, as well as iron acquisition, capsule

synthesis, and pColV plasmid markers encoding genes. Hybridization to the outer membrane protein gene involved in the pyelonephritis-associated pili assembly (*papC*), the variants of fimbrial subunit of P pili (*papA*), and/or the pili adhesin (*papG*) were used to identify a cluster of 13 strains having the genetic potential to colonize urethra, bladder, and kidney. These strains harboured genes related to the constant region of pColV plasmid, including the increased serum survival factor (*iss*), the complement resistance protein (*traT*), the aerobactin operon, *iucD* (in 10 of 13 strains), and the siderophore receptor,

Table III. *Virulence factors characterizing ExPEC*. Prevalent profiles of UPEC/APEC strains.

Strain*	Sero-type	Phylo-type	Fimbrial factors	Toxins	Adhesins, invasins	Iron acquisition	Capsule synthesis	pColV determinants
1-A	O121	B1	*papA(11), papGII, papC, f165A(1)*	*vat, senB, ccdB*	*hra1, tibC, ibeB*	*fepC, fyuA, iroN, iucD, sitAD, iutA*	*kpsM-III*	*iss, traT, tsh, cvaC, cba, cei, cma, col5*
1-B	NT	B2	*papA(11), papGII, papC, f165A,*	*hlyE, vat*	*hra1, ibeB*	*chuA, iroN, fepC, fyuA, iucD, iutA, sitAD*	*kpsM-II, neuA, neuC*	*iss, traT, ompT, tsh, cvaC, cia*
1-C	O111	B2	*papA(11), papGII, papC, f165A(1)*	*hlyE, senB, vat*	*hra1, ibeB, tibC*	*chuA, fepC, irp1, 2, fyuA, iroN, iucD, iutA, sitAD*	*kpsM-II, neuAC*	*iss, traT, tsh, ompT, cvaC, cei, cia, cma, col5*
1-D	NT	B2	*papA(11), papGII, papC, f165A*	*hlyE, vat*	*hra1, ibeB, tibC*	*chuA, fepC, irp1, 2, fyuA, iroN, iucD, iutA, sitAD*	*kpsM-II, neuAC*	*iss, traT, ompT, tsh, cvaC, cia*
4-B	NT	B1	*papA(11), papGII, papC, f165(1)A, flmA54*	*hlyE, ccdB*	*hra1, ibeB, tibC,*	*iroN, iucD, iutA, sitAD,*		*iss, traT, tsh, cvaC, cba, cei, cma*
9-A	O86	B1	*papA(7-2, 11), papC, F17a, f165(1)A, fimF41A*	*hlyE, ccdB*	*hra1, ibeB, afaD, afaE, nfaA,*	*fepC, fyuA, iucD, iutA, sitAD*		*iss, traT, ce1a, cei*
10-C	O8	B1	*papA(12), papC, papGII, F17c*	*hlyE, ccdB*	*hra1, ibeB, afaDE, tibC*	*fepC, irp2, iroN, fyuA, iucD, iutA, sitAD*	*kpsM-III*	*iss, traT, shf, cvaC, col5, cba, cia, cma*
10-F	O155	B1	*papA(12), papC, papGII, F17c*	*hlyE, senB*	*hra1, ibeB, afaD, tibC, nfaA*	*fepC, iroN, fyuA, sitD*		*iss, traT, cvaC col5, cei, cba, cib, cma*
12-D	O56	D	*papA(7-2, 14, 15), papGIII, F17a, fimF41a, f165(1)A*	*hlyAE, senB, cdtB(2, 3, 4), ccdB*	*afaDE, ibeAB, tibC, daaE, nfaA*	*chuA, fepC, irp1, 2, fyuA, iutA, sitAD*		*iss, traT, tsh, cvaC, col5, colY, cda, cia, cei*
12-E	O104	D	*papA(7-2, 11, 14, papGII, pixA, sfaD, fimF41A*	*hlyE, senB, cdtB(1, 2, 2/3, 3, 4), ccdB*	*IbeB, afaDE, tibC, daaE, nfaA, drbE,*	*chuA, fepC, irp2, fyuA, iroN, iutA, iucD, sitAD*	*kpsM-II*	*iss, traT, ompT, cvaC, col5, cei, cia, ce1a*
12-F	O155	B1	*papA(7-2, 14, 15), papC, F17b, fimF41A, sfaD, f165(1)A*	*hlyE, cnf1, 2, cdtB(2)*	*ibeB, afaDE*	*fepC, irp1, fyuA, iroN, iucD, iutA, sitAD*	*kpsM-III, neuA*	*iss, traT, ompT, cvaC, cia*
13-A	O55	B1	*papA(11), papC, papGI, f165(1)A*	*hlyAE, cnf2, cdtB(3), ccdB*	*hra1, afaDE ibeB, daaE, drbE*	*iucD, iutA,*		*iss, traT, ompT, cvaC, col5, cia, cei*
14-A	O155	B1	*papA(7-2, 8, 9, 14), papC, F17d*	*hlyE, cnf1, ccdB*	*hra1, ibeB, afaE, drbE*	*fepC, fyuA*	*kpsM-III*	*traT*

*Strain identification was coded by Farm number (from Figure 1) and letter (strain isolate); NT = nontypeable.

Table IV. *Virulence factors characterizing ExPEC*. Prevalent profiles of APEC strains.

Strain*	Sero-type	Phylo-type	Fimbrial factors	Toxins	Adhesins, invasins	Iron acquisition	Capsule synthesis	pColV determinants
3-C	O86	B1	*papA(7-2, 14), F17a*	*hlyE, senB*	*ibeB, afaDE, tibC, nfaAE*	*fepC, irp2, fyuA,*		*iss, traT, cei, col5*
7-E	NT	D		*hlyE,*	*ibeB, afaD, tibC*	*chuA, fepC, iroN, iucD, iutA, sitAD,*	*kpsM-II,*	*iss, ompT, cvaC, cia*
9-B	NT	B1	*papA(7-2, 14), flmA54*	*hlyE, ccdB*	*ibeB, afaE5, tibAC*	*fyuA, iutA*		*iss, ompT, traT, tsh*
11-C	O146	NT	*papA(7-2, 14), F17b*	*hlyAE, cnf2, senB*	*hra1, ibeA, afaDE, nfaA,*	*fepC, irp2, fyuA, iucD, iutA*		*iss, traT, col5, cda, cia, csa*

*Strain identification was coded by Farm number (from Figure 1) and letter (strain isolate); NT = nontypeable.

Table V. *Virulence factors characterizing ExPEC*. Prevalent profiles of atypical APEC strains.

Strain*	Sero-type	Phylo-type	Fimbrial factors	Toxins	Adhesins, invasins	Iron acquisition	Capsule synthesis	pColV determinants
4-C	NT	B2		*hlyE, ccdB*	*hra1, ibeAB, tibC*	*chuA, fepC, irp2, fyuA, iucD, iutA, sitA, sitD*		*traT, ompT, tsh, cvaC, cba, ce1a, cia, cma*
6-A	O26	B1	*papA(11), papC, f165(1)A*	*hlyE, ccdB*	*hra1, ibeB, tibC*	*iucD, iutA, sitA, sitD*		*traT, ompT, cda, ce1a, cei*
10-A	NT	D	*papA(12), papGI, papGIV, focG, F17bc*	*hlyE, ccdB*	*hra1, afaDE*	*chuA, fepC, irp2, iucD, iutA, sitA, sitD*		*caa, cma*
10-B	O45	D	*papA(7-2, 14), papGI*	*hlyE, senB, ccdB*	*ibeB*	*chuA, fepC, iucD, iutA, sitA, sitD*	*kpsM-II, neuA*	*ompT cna*
11-D	O155	B1	*papA(7-2, 14)*	*hlyE, ccdB*	*IbeB, afaE*	*fyuA, iutA*		*traT, ompT*
13-B	O155	NT			*tibC*	*sitA*	*kpsM-III, neuA*	*col5, cba, cei, cda, cna, csa, ce1a*

* Strain identification was coded by Farm number (from Figure 1) and letter (strain isolate); NT = nontypeable.

Table VI. *Virulence factors characterizing undefined ExPEC strains.*

Strain*	Sero-type	Phylo-type	Fimbrial factors	Toxins	Adhesins, invasins	Iron acquisition	Capsule synthesis	pColV determinants
10-E	O8	NT	*F17ac*	*hlyE,*	*hra1, ibeB, afaD,*			*Iss*
11-A	NT	NT		*hlyE*	*ibeB*			*iss*
11-B	NT	NT	*papA(14)*	*hlyE, senB*	*ibeB*			*traT*

* Strain identification was coded by Farm number (from Figure 1) and letter (strain isolate); NT = nontypeable.

iroN (9 of 13 strains). This virulence gene pattern classified them as UPEC/APEC strains (Table III). Among the remaining 13 strains, those that were missing or possessing a partial set of P pili-encoding genes but showing the *iss* gene and genes involved in iron uptake were classified as APEC (Table IV). Finally, a cluster of ExPEC strains were characterized by a genetic profile composed of a pColV plasmid, iron acquisition system, but lacked the *iss-iroN* pattern or harboured the *iss* gene, but had an overall weak positive signal for virulence genes. These strains were classified as atypical APEC (Table V) or as undefined (Table VI) strains, respectively.

Discussion

The microarray used in this study is a useful tool to sort *E. coli* strains into various classifications (serogroup, phylogenetic group, and pathotype), including identifying common virulence plasmids. Stool sampling only partially limited the diagnostic value of this technique with respect to direct isolation from intestinal contents. In fact, only the LEE and TTSS-harbouring strains (about 50%) could be considered as the cause of diarrhoea in calves. The aEPECs (Table I) showed the combination of LEE gene variants of the D group of Afset (Afset *et al.* 2008), which classified human diarrhoeagenic aEPECs into 11 groups (A to K group) based on LEE genes variants, namely e*spA, espB, tir*, and *eae*. The 2 combinations of LEE variants found in the O26- and O111-EHEC strains, respectively, belonged to groups D and B of Afset. The third LEE variant combination, *espA2, espB1, tir-3, eae(gamma2)*, related to 3 strains (O157-, O155-, nontypeable-EHEC), was not found in the Afset classification. The *eae(beta)* variant characterizing serotype O26 EHEC strains confirmed a previous study on gene profile patterns in diarrhoeagenic calves in the province of Tehran (Badouei *et al.* 2010). All but 2 InPEC strains contained genetic markers related to colonization (*efa1/lifA*); while cell effectors (*nleB, nleE, nleF, nleG*) have been recently considered markers in the molecular risk assessment for virulent EPEC and EHEC in human colibacillosis (Zang *et al.* 2007, Afset *et al.* 2008, Bugarel *et al.* 2011). The microarray used in the present study did not detect the *arpA* gene, encoding the ankyrin repeat protein, considered in the revised Clermont phylogrouping method. This gene, common in avirulent *E. coli* strains, could have helped to better differentiate the 4 phylogroups considered in this study, including the possible assignment to phylogroup E (mostly O157:H7), F (a sister group of B2), and C, closely related to B1 strains (Clermont *et al.* 2013).

In contrast, the variability and abundance of virulence patterns in a large group (at least 23 strains out of 26) of ExPEC-typed strains revealed the epidemiological value of our microarray technology when diarrheal stools are investigated. In general, the virulence gene profile of these strains was complex. Our ExPEC strains contained 1 or more

pVir plasmid products known to cause human and bovine septicemia (Lopez-Alvarez and Gyles 1980, El Mazouari *et al.* 1994), including genes coding for cytotoxic necrotizing factor 2 (*cnf-2*), type III cytolethal distending toxin (*cdtB-1,2,2/3,3,4*), and *F17abcd* fimbriae. Furthermore, *tibAC* adhesin/invasin genes, involved in bacterial aggregation and biofilm formation, are diffusely present in UPEC/APEC-typed strains. Moreover, several of these strains also harboured the Dr family adhesins (*daaE, nfaAE* and *drbE* genes), the Prs-like fimbriae (*f165* and *focAG* genes), and the genetic markers of pAA or pAA2 plasmids (strains 9B, 10A, 10C, 12D, and12E; data not shown), reflecting a high level of genomic plasticity among these strains (see below).

The diffuse distribution of pColV markers, among the UPEC/APEC strains (Table III, IV and V) could reflect their avian origin. However, the prevalent avian colibacillosis-associated O1, O2 or O78 serotypes (Kabir 2010, Schouler *et al.* 2012) were not found in the examined strain collection. The pColV-harbouring strains 1A, 12D, 12E, and 13B showed multidrug resistance (MDR) plasmid patterns comprising aminoglycosides, sulfonamides, quinolones, and the Extended Spectrum Beta-Lactamase (ESBL) CTX- and TEM-types (Badagliacca *et al.* 2014, data not shown). The linkage of MDR-encoding regions with the pColV plasmid was investigated, since it provided a mean for the selection of virulent strains through the use of antibiotics (Johnson and Nolan 2009, Brzuszkiewicz *et al.* 2011).

The inherent genomic plasticity of *E. coli* was suggested as a key to understand the thin interface between commensal and human/animal (avian) pathogenic strains in the pathotyping of ExPEC isolates (Escobar-Paramo *et al.* 2006, Rendón *et al.* 2007, Clermont *et al.* 2011, Schouler *et al.* 2012). The 'pangenome' of *E. coli* is considered a genomic mosaic between a pathogen and a commensal, which evolves by gene acquisition and diversification as revealed by Rasko and colleagues (Rasko *et al.* 2008) following the genomic sequence of a commensal *E. coli*. These findings further support the idea that this dynamic genome plasticity, caused essentially by the dynamic exchange of virulence genes through mobile genetic elements like plasmids or pathogenicity islands, may highlight an evolutionary strategy for *E. coli*, namely the creation of a mixed assortment of virulence factors coming from various pathogenic strains (Schubert *et al.* 2009). This strategy could lead to serious problems in public health through the emergence of highly virulent new strains as it was dramatically illustrated by the 2011 Shiga toxin-producing *E. coli* O104:H4 in German and France outbreaks (Denamur *et al.* 2011, Scheutz *et al.* 2011, Rasko *et al.* 2011, Brzuszkiewicz *et al.* 2011, Zhang *et al.* 2013).

In conclusion, the microarray method has proven to be an easy, robust tool for the pathotyping of *E. coli* isolates. In particular, it was suited to accurately type attaching and effacing *E. coli* (AEEC-types). Moreover, the microarray platform has provided a database for the meta-analysis of ExPEC-typed strains from diarrheal stools of calves. This database should prove useful for further analysis of gene expression (transcriptomics, RNA-seq) or for the development of predictive computational models of the biological or pathogenic significance of gene products found in commensal strains of *E. coli*. As mentioned above, due to rapid virulence gene movement among *E. coli* strains, the emergence of new pathotypes (Denamur *et al.* 2011, Brzuszkiewicz *et al.* 2011) or multiple pathotypes (Bekal *et al.* 2003) can occur. Simultaneously screening for such a large, comprehensive number of virulence and virulence associated genes through microarray platforms could allow for detecting novel pathogens that are easily missed by more conventional PCR methods.

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