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Identification and detection of three new F17 fimbrial variants in *Escherichia coli* strains isolated from cattle

Morgan Bihannic¹²³, Reza Ghanбарpour⁴, Frédéric Auvray⁵, Laurent Cavalié⁶, Pierre Châtre³, Michèle Boury¹², Hubert Brugère¹², Jean-Yves Madec³* and Eric Oswald¹²³⁶

**Abstract**

F17 fimbriae are produced by pathogenic *Escherichia coli* involved in diarrhea and septicemia outbreaks in calves and lambs. These proteins result from the expression of four different clustered genes, namely f17A, f17D, f17C and f17G, encoding a pilin protein, a periplasmic protein, an anchor protein and an adhesin protein, respectively. Several variants of f17A and f17G genes have been reported and found genetically associated with typical virulence factors of bovine pathogenic *E. coli* strains. In this study, a new F17e-A variant, closely related to F17b-A, was identified from a collection of 58 *E. coli* isolates from diarrheic calves in Iran. While highly prevalent in Iranian F17-producing clinical isolates from calves, this variant was rare among *E. coli* from a French healthy adult bovine population, suggesting a possible association with virulence. The f17Ae gene was also found in the genome of the Shiga-like toxin variant Stx₁d-producing bovine *E. coli* strain MHI813, and belonged to a gene cluster also encoding a new F17-G3 variant, which greatly differed from F17-G1 and F17-G2. This gene cluster was located on a pathogenicity island integrated in the tRNA *pheV* gene. The gene coding for a third new F17f-A variant corresponding to a combination of F17c-A and F17d-A was also identified on the pVir68 plasmid in the bovine pathogenic *E. coli* strain 6.0900. In conclusion, we identified three new F17-A and F17-G variants in cattle *E. coli*, which may also have significant impact on the development of new diagnostics and vaccination tools.

**Introduction**

*Escherichia coli* is a predominant member of the normal aerobic intestinal microflora in mammals. However, due to the high genetic plasticity of the *E. coli* species, some *E. coli* strains may behave as pathogens and be responsible for a wide range of infections. These infections can be split into intestinal and extra-intestinal infections, such as urinary tract infection (UTI), meningitis or septicemia [1,2]. In veterinary medicine, different pathotypes of *E. coli* have been recognized, such as Avian Pathogenic *E. coli* (APEC) in birds or Enterotoxigenic *E. coli* (ETEC) in cattle. For pathogenic isolates, adhesion to host cells is an essential step before colonization and possible invasion, and these strains usually produce fimbrial or afimbrial adhesins to bind host cells receptors. In ruminants, some of them are involved in diarrhea (intestinal disease) or septicemia (extra-intestinal disease) outbreaks, particularly in newborn calves and lambs [3,4], and produce fimbriae of the F17 family. Despite an obvious association with virulence, the exact role of F17 fimbriae in the pathogenicity of *E. coli* remains unknown. These fimbriae were reported to bind N-acetyl-D-glucosamine (Glc-NAc)-containing receptors present on host intestinal epithelial cells in bovines [4,5]. They consist in fine filamentous heteropolymers composed of two main subunits: the structural major subunit F17-A, whose hundred copies are assembled to form the bulk of the fimbriae, and the adhesin minor subunit F17-G [4]. Among the F17 fimbriae family, several variants of the two subunits have been identified. This polymorphism was shown to result in changes in receptor specificity, in association with various virulence factors and clinical disorders [3]. Four variants of the major subunit F17-A are currently known: F17a-A, formerly named FY antigen and...
characterized in bovine ETEC strains [6]; F17b-A, formerly named Vir antigen and identified in *E. coli* strains responsible for diarrhea or septicaemia in calves [7]; F17c-A, formerly called 20 K and associated with *E. coli* strains responsible for diarrhea or septicemia in calves [8]; and F17d-A, previously known as F111 and identified in bovine ETEC [9]. Two variants of the minor subunit F17-G have been identified: F17-G1 and F17-G2 [10], that are randomly associated with the 4 different variants of the subunit F17-A [11]. The F17 fimbriae are also expressed by a few human uropathogenic *E. coli* strains [12], and were described in these strains as G fimbriae [13], with two subunits GafA and GafD, that correspond to the variants F17c-A and F17-G2, respectively. Some of these variants are known to be associated with virulence factors involved in bovine pathogenesis, like CNF2 (cytotoxic necrotizing factor 2) or CDT-III (cytotoxic lethal distending toxin III) toxins [10,14,15], and the detection of these virulence factors and of F17 fimbriae is used to monitor their genetic flux and association. Two methods are widely used for the detection of F17 variants, i.e. immunological detection using specific anti-F17-A antibodies - no F17-G antibodies have been reported to date -, and PCR using either specific primers for the F17-A and F17-G variants encoding genes or consensual primers for the F17-A encoding genes family [8,10]. In F17-positive strains, negative PCR results with specific primers of every known F17 variant revealed the likely existence of unknown F17 variants [10].

In this study, we report the characterization of two new variants of the structural subunit F17-A in calves, that we propose to name F17e-A and F17f-A. A new variant of the F17-G adhesin subunit, proposed as F17-G3, was also identified, in association with the new variant F17e-A on a pathogenicity island. Based on specific primers designed in this study, we also report a very weak prevalence of F17e-A and F17-G3 in *E. coli* from healthy adult cattle. Lastly, a widely used F17-producing isolates detecting antiserum was tested on reference strains for each variant of the major subunit and was found to detect the variant F17a-A only.

**Materials and methods**

**Bacterial strains and growth conditions**

*E. coli* strain MHI813 (ONT:H19 serotype) was isolated from a healthy bovine feces and shown to produce the new Stx1d variant of Shiga Toxin Stx1 [16]. *E. coli* strain 6.0900 (O36:H5 serotype) was isolated from feces of a calf with diarrhea and shown to harbor the pVir68 plasmid [17]. *E. coli* strains 25K9H9, 55, 31A and 111K9H86 (6-9] were used as positive controls for F17a-A, F17b-A, F17c-A and F17d-A variants, respectively (Table 1). All strains were grown in Luria-Bertani (LB) broth (Invitrogen, Paisley, Scotland) for 16 h at 37 °C with 220 rpm shaking. For FY antiserum testing, *E. coli* isolates were grown overnight on polyvitaminic supplemented Minca medium (BioMérieux, Marcy l’Etoile, France) at 37 °C.

**Collection of *E. coli* isolates from diarrheic calves in Iran**

Between March 2004 and June 2005, a collection of 58 *E. coli* isolates was recovered from rectal swabs of 58 diarrheic calves aged 3 to 14 days old, bred in 11 herds in the Kerman province, Southeastern Iran. The 11 herds were either in industrial or traditional rearing conditions without any history of antibiotherapy. Rectal swabs were immediately sent out to the laboratory in ice-cooled containers and streaked on MacConkey agar.

**Table 1** Strains used in this study

| Bacteria | Strain | Fimbriae | F17-A-like subtype | F17-G-like subtype | Host | Pathology | Reference |
|----------|--------|----------|-------------------|-------------------|------|-----------|-----------|
| *E. coli* | 25K9H9 | F17      | F17a-A            | F17-G1            | Calf | Diarrhea  | [6]       |
|          | S5     | F17b-A   | F17-G2            |                    | Lamb | Septicaemia| [7]       |
|          | 31A    | F17c-A   | F17-G2            |                    | Calf | Septicaemia| [8]       |
|          | 111K9H86 | F17d-A   | F17-G1            |                    | Calf | Diarrhea  | [9]       |
|          | MHI813 | F17e-A   | F17-G3            |                    | Cow  | No path.  | [16]      |
|          | 6.0900 | F17f-A   | F17-G2            |                    | Calf | Diarrhea  | [17]      |
|          | CIK210 | F17      | F17a-A            | F17-G2            | Lambs and goats kids | Diarrhea | [11]      |
|          | CIK377 | F17c-A   | F17-G2            |                    |      |           |           |
|          | CL114  | F17d-A   | F17-G2            |                    |      |           |           |
|          | CL394  | F17d-A   | F17-G1            |                    |      |           |           |
|          | IHI1165| GaFA     | GaFD              |                    | Human| Cystitis  | [13]      |
|          | 536    | F17-related | F17-A 536 | F17-G 536         | Human| Cystitis  | [18]      |
| *P. mirabilis* | HI4320 | UcaA | UcaA | UcaG | Human | Cystitis | [19]      |
|          | HU1069 | UcaA     | UcaG              |                    |      |           | [20]      |
| *B. cereus* | ATCC14579 | Bcp | BcpA | BcpB | Human | Numerous | [21]      |
plates (Biolife Laboratories, Milan, Italy), which were incubated overnight at 37 °C. A single lactose-positive colony from the fecal culture from each calf was selected and confirmed biochemically to be E. coli (API 20E, BioMérieux, Marcy l’Étoile, France) and stored at −80 °C in LB broth with 30% sterile glycerol. After DNA extraction, virulence-associated genes were detected by PCR with specific primers of the aap, aggR and AA probe genes [22], f17A genes family, f17Aa, f17Ab, f17Ac, f17Ad, f17G1 and f17G2 genes [10], afaE-VIII gene [23], F5 and F41 fimbriae encoding genes [24], stx1, stx2 and eae genes [25], ltl, stl and ipaH genes [26], pap E-F, afaI (B-C), sfa, hly and iucD encoding operons [27], cnf1, cnf2, cdto1 and cdto4 genes [28] and clpG gene [29]. The primers used and expected sizes of PCR products are presented in Table 2. The PCR products were detected by 2% agarose gel electrophoresis and 3X GelRed staining.

The localization of the F17 gene cluster on the genome of E. coli isolates was determined with 24 antisera provided by LREC, University of Santiago de Compostela, Lugo, Spain. Phylogenetic groups (A, B1, B2, or D) were determined by triplex PCR [30] and the combinations of three genetic markers clpA, yjaA, TspE4.C2 were used for phylogenetic subgroups determination (A0, A1, B1, B2, B2, D1 and D2) [31]. Clonality of isolates was investigated by Pulse-field gel electrophoresis (PFGE) analysis. In brief, whole-cell DNA from E. coli isolates was digested with XbaI (for 16 h at 37 °C) after lysis by lysozyme and proteinase K. Electrophoresis of enzyme-generated fragments was performed with a contour-clamped electric field CHEF Mapper XA System apparatus (Bio-Rad) through a 1% agarose gel. Migration was performed for 30 h at 14 °C, with an electric field of 6 V/cm. DNA bands patterns were visualized with ethidium bromide, digitally photographed and analyzed with Gelcompar II 6.5 software. DNA bands size was determined from CHEF DNA Size Standard, Lambda Ladder (Bio-Rad) (Size range: 50–1000 kb).

In silico analysis of E. coli MH8183 genome and plasmid pVir68

The localization of the F17 gene cluster on the genome of E. coli MH8183 was determined by nucleotide basic local alignment search tool (BLASTn) analyses on E. coli MH8183 whole genome shotgun (wgs) sequences (taxonomy ID: 754089, GenBank:AFDZ010000000.1) using the nucleotideic sequences of the already described F17 variants. The genetic environment of the F17 cluster on the genome was determined using BLASTn analysis of E. coli MH8183 wgs sequence carrying F17 cluster on the entire nucleotide collection database. All automatic annotations of coding sequences located in the vicinity of the F17 cluster were also checked with Artemis software (Wellcome Trust Sanger Institute, Hinxton, UK) and BLASTX analyses. The F17 cluster on pVir68 plasmid sequence (GenBank accession number: CP001162.1) was analyzed by BLASTn and f17A gene on pVir68 sequence was compared with f17Ac and f17Ad genes from 31A and 111K86 reference strains by alignment with ClustalX2 software.

Phylogenetic analysis of F17-A and F17-G variants encoding genes

For F17-A and F17-G phylogenetic assessment, all nucleotideic sequences of F17-A and F17-G variants were obtained from GenBank [AF022140.1, L14318.1, L14319.1, L43373.1, L43374.1, L77091.1, AFDZ01000020.1, CP001162.1, AF055306.1, AF055307.1, AF055308.1, AF055309.1, AF055310.1, AF055311.1, AF055312.1, AF055313.1, L39691.1, L43372.1, CP000247.1, U28420.1, and AM942759.1]. The sequences corresponding to the fimbrial-encoding genes BcpA and BcpB from Bacillus cereus strain ATCC14579 [GenBank:AE016877.1] were used as outgroups for phylogenetic analyses of f17A genes and f17G genes respectively. The sequences were aligned with MEGA 5.2 software. Based on the F17 variants alignments, phenograms were drawn using the Maximum Likelihood method based on the Tamura-Nei model [32].

F17e-A and F17-G3 variants PCR detection

Two primers were designed with Primer3Plus software for specific PCR detection of the new variants F17e-A and F17-G3: 5′-CTGACTCCATTACCTTGAGC-3′ for F17e-A and 5′-GCAATAATGGTGGATATGAAACA-3′ for F17-G3. These primers were set up in addition to the currently available multiplex PCR protocol for the detection of all known variants of F17-A and F17-G [10].

F17e-A and F17-G3 prevalence in a healthy bovine population in France

A feces sampling campaign was performed on 787 healthy adult bovines entering in 6 slaughterhouses in Western France from October 2010 to March 2011. After enrichment in trypticase-soy novobiocine added broth overnight at 37 °C, the total DNA was extracted with the Food Extraction Pack kit (GeneSystems). A F17 screening was performed on the total DNA samples extracted from each bovine feces sample by qPCR with F17 common primers. On the F17-positive samples identified, F17e-A and F17-G3 were searched using specific primers as described above.

FY antiserum testing on F17A variants reference strains

The FY antiserum (Biovac, Angers, France) was assessed by agglutination test on E. coli strains 25KH9, S5, 31A, 111K86, MH8183 and 6.0900, which were used as reference strains for F17a-A, F17b-A, F17c-A, F17d-A, F17e-A and F17f-A variants respectively.
Table 2 Oligonucleotide primers used for detection of the virulence-associated genes on E. coli isolates from feces of calves with diarrhea in Iran

| Gene/DNA fragment | Primer sequence (5′ - 3′) | Product size (bp) | Reference |
|-------------------|---------------------------|-------------------|-----------|
| *aoa*             | GCTTGGTGCTACGCCTGAAATG    | 310               | [22]      |
|                   | AACCCTTGGGTGATGGCACC      |                   |           |
| *aggR*            | CTAATTGTACAAATGATGTA      | 457               | [22]      |
|                   | AGAGTCCATCTCTTGATAAG       |                   |           |
| *AA probe*        | CCTGGCGAAAGAAGCTCATCAT    | 629               | [22]      |
|                   | CAATGTATAGAAATCCGCTGTG    |                   |           |
| *fi7A family*     | GCAGAAAAATTCATTATCCCTGGG  | 537               | [10]      |
|                   | CTGATAACGCAGTGTAAATTAAC (P2)*  |                   |           |
|                   | GCTGGAAGGGTGCAAATACGCCGT  | 321               | [10]      |
|                   | CATACCAACGGGATAGCTACAGTTTC| 323               | [10]      |
|                   | GCAAGGAACCGGCTCCCTGGG     | 416               | [10]      |
|                   | GATAGTCATAACCTTATATGCA     | 239               | [10]      |
|                   | CGGAGCTAAATCAGCAGCACC     | 615               | [10]      |
|                   | TGTGTATACCGGTTAACGGTAC     |                   |           |
|                   | CGTGGGGAAAATATCATACGACG    | 615               | [10]      |
|                   | TGTGTATACCGGTTAACGGTAC     |                   |           |
| *afa E-VIII*      | CTAACCTGGCCATGCTGACAGTA   | 302               | [23]      |
|                   | TTATCCTTCTCGTGATG          |                   |           |
| *f5*              | TATATCTTAGGGCTGATTG        | 314               | [24]      |
|                   | GGTATCCCTTTACGACAGATTACTTC|                   |           |
| *f41*             | GCATCAGCGGCGATGCTCGGATG   | 380               | [24]      |
|                   | GTCCCTAGCTCAGATATTACACCT   |                   |           |
| *stx1*            | AGACCGGTAGTTACGGTTG       | 388               | [25]      |
|                   | TTGGCCACGAGTGGATG          |                   |           |
| *stx2*            | TGCTTTTCTTCTCGGATC         | 807               | [25]      |
|                   | GACATTCTGGGCTGACTCTCTTT    |                   |           |
| *eaeA*            | AGGCCTCGTCAACAGTTG         | 570               | [25]      |
|                   | CACATCGTCCACAGAGGA         |                   |           |
| *stl*             | ATTTTTGTTTCTGATTATTCTT     | 190               | [26]      |
|                   | CACCCGGTACARGCAGGATT       |                   |           |
| *hly*             | GGCGACAGATTACCCGGTG       | 450               | [26]      |
|                   | CGGTCTCTATATCCCTGTT        |                   |           |
| *ipaH*            | GTCCTTTGACCGCCTTCCGGCATACGGTC| 600               | [26]      |
|                   | GCCGCTACGGCCTCTGAGGATCAC  |                   |           |
| *popE-F*          | GCAACACAGCAAGCCTGTTGACATCAT| 336               | [27]      |
| *afr B-C*         | GCTGGGACAACTTGATACACTCTCCTC| 750               | [27]      |
|                   | CATCAACGTGTCTTGCTCCGGCG   |                   |           |
| *sfa/focDE*       | CGGAGAGTAATACAAACCTGGCA   | 410               | [27]      |
|                   | CTCGGGAGAACTGGGCTGACATTCTAC|                  |           |
| *hly*             | AACAAGGATAAGCACTGCTCCTGGC | 1177              | [27]      |
|                   | ACCATATAACGCCGTATTCCGGTCA  |                   |           |
Results
A new F17e-A variant of the F17 fimbriae major subunit was detected in diarrheic calves in Iran
Among the 58 E. coli isolates recovered from diarrheic calves in Iran, various virulence-associated genes were identified, such as the hly, iucD, iucD, or afaE-VIII genes (Table 3). Interestingly, 20 out of 28 F17-A-positive isolates (71.4%), which were also shown to carry the F17-G2 variant, could not be further characterized using specific PCR for the F17-A variants. These isolates originated from 7/11 herds and corresponded to 5 E. coli clones (I to V), as shown by PFGE (Figure 1). Clone I and II were closely related with 90% identity on their PFGE patterns and included 11 ONT and 2 ONT and 1 O101 isolates respectively. Clone III included 3 ONT isolates, one of them carrying the enterotoxigenic E. coli characteristic genes LT-I and ST-I, clone IV included a single O15 isolate and clone V included 2 ONT isolates, which were found to carry the typical bovine necrotoxigenic E. coli genes cnf2 and cdtIII [33,34]. The PCR product obtained with F17-A common primers on one E. coli isolate of the most prevalent clone (clone I) was sequenced and compared to the F17-A variants sequences by ClustalX2 software alignment. The F17-A PCR product sequence was found to share from 73 to 93% identity with the sequences of the four already described F17-A variants, suggesting that it was corresponding to a new variant that we named F17e-A. A specific forward primer for this new variant was designed to be used with the common reverse primer P2, which is used for detection of the f17A family, f17Aa, f17Ab, f17Ac, and f17Ad genes [10]. A PCR with the F17e-A specific primer was then performed on all other 19 uncharacterized F17-A E. coli isolates, that were found to carry the new variant F17e-A. The F17e-A variant is encoded on a gene cluster carried by a pathogenicity island on E. coli MHI813 genome, in association with a new variant F17-G3 of the minor subunit
The MHI813 strain was previously detected to be positive with F17-A common primers PCR but tested negative with F17a-A, F17b-A, F17c-A, and F17d-A specific PCRs as well as with F17-G1 and F17-G2 specific PCRs (data not shown). However, the MHI813 strain was now found positive using the newly designed F17e-A specific primer. To identify the localization and the genetic environment of the f17Ae gene on MHI813 genome, the wgs sequence of MHI813 strain was used. The in silico analysis of MHI813 genome revealed that the F17 fimbriae encoding genes, ie f17A, f17D, f17C and f17G, were organized in a gene cluster on the contig 19 of MHI813 strain wgs sequence. The N-terminal half of the adhesin encoding gene f17G on MHI813 genome also showed no identity with the same part of the adhesin genes of the other variants. This result suggests the expression of a very different adhesin in MHI813 strain, that we named F17-G3, and for which we developed a specific primer for its detection by PCR. The nucleotide sequences of f17Ae and f17G3 genes

Table 2 Oligonucleotide primers used for detection of the virulence-associated genes on E. coli isolates from feces of calves with diarrhea in Iran (Continued)

| Gene | Forward primer | Reverse primer | Length (bp) | Reference |
|------|----------------|----------------|-------------|-----------|
| iucD | TACCGGATTGTCCATATGCAGACCG | P2 | 602 | [27] |
| cnfI | GGGGGAAGTACAGAAGAATTA | TTTGTCGGTGGCAGCAGGGAAGAAA | 1111 | [28] |
| cnfII | TATCATAGGCCAGGGAGGACACC | GTCAACAATAGAAATTTTTCGG | 1240 | [28] |
| cdtIII | GAAATAAAATGGAATATAAATGTCCG | TTTGTGTCGGTGGCAGCAGGGAAGAAA | 555 | [28] |
| cdtIV | CCTGATGGTTAGAGGCTGGTTTC | TTGCTGCAGAATCTATACCT | 350 | [28] |
| clpG | GGGCGCTGCTGCTCTCAAC | CGCCCTAATGCTGGCGAC | 402 | [29] |
| chuA | GACGAACCAACGTCAGGAT | TGCCGGCCAGTACAAAGACA | 279 | [30] |
| yjaA | TGAAGTGCAGGAGCCTGTC | ATGGAGAATGCGTTCCTCAAC | 211 | [30] |
| TspE4.C2 | GAGTAATGTGGGACCGATTTCA | CGCCGCAACAAAGATTTACG | 152 | [30] |

*P2 was used as common reverse primer for f17A family, f17Aa, f17Ab, f17Ac, and f17Ad detection.

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http://www.veterinaryresearch.org/content/45/1/76
Table 3 Detection and identification of F17 genes variants and virulence-associated genes in the collection of *E. coli* isolates from feces of calves with diarrhea in Iran

| Isolate | Sampling date | Serogroup | Phylogroup | F17A | F17a-A | F17b-A | F17c-A | F17d-A | F17e-A | F17-G1 | F17-G2 | Other virulence-associated genes |
|---------|---------------|-----------|------------|------|-------|-------|-------|-------|-------|-------|-------|---------------------------------|
| 2       | March-04      | -         | B1-1       |      |       |       |       |       |       |       |       |                                 |
| 10      | March-04      | -         | B1-1       | +    |       |       |       |       |       |       |       |                                 |
| 30      | May-04        | -         | B1-1       | +    |       |       |       |       |       |       |       |                                 |
| 44      | May-04        | O8        | B1-1       |      |       |       |       |       |       |       |       |                                 |
| 38      | May-04        | -         | B1-1       | +    |       |       |       |       |       |       |       |                                 |
| 49      | June-04       | -         | B1-1       |      |       |       |       |       |       |       |       |                                 |
| 52      | June-04       | O8        | B1-1       |      |       |       |       |       |       |       |       |                                 |
| 64      | March-05      | O157      | A-1        |      |       |       |       |       |       |       |       |                                 |
| 16      | April-04      | -         | B1-1       |      |       |       |       |       |       |       |       |                                 |
| 68      | April-05      | -         | B1-1       | +    |       |       |       |       |       |       |       |                                 |
| 73      | June-05       | -         | D-1        |      |       |       |       |       |       |       |       |                                 |
| 45      | May-04        | -         | B1-1       |      |       |       |       |       |       |       |       |                                 |
| 57      | June-04       | O15       | B1-1       | +    |       |       |       |       |       |       |       |                                 |
| 4       | March-04      | O8        | B1-1       |      |       |       |       |       |       |       |       |                                 |
| 3       | March-04      | O8        | B1-1       |      |       |       |       |       |       |       |       |                                 |
| 29      | April-04      | -         | B1-1       | +    |       |       |       |       |       |       |       |                                 |
| 56      | June-04       | O8        | A-1        |      |       |       |       |       |       |       |       |                                 |
| 25      | April-04      | -         | B1-1       | +    |       |       |       |       |       |       |       |                                 |
| 35      | May-04        | -         | B1-1       | +    |       |       |       |       |       |       |       |                                 |
| 63      | March-05      | O49       | B1-1       |      |       |       |       |       |       |       |       |                                 |
| 5       | March-04      | -         | B1-1       | +    |       |       |       |       |       |       |       |                                 |
| 26      | April-04      | -         | B1-1       | +    |       |       |       |       |       |       |       |                                 |
| 28      | April-04      | O101      | B1-1       | +    |       |       |       |       |       |       |       |                                 |
| 32      | May-04        | -         | B1-1       | +    |       |       |       |       |       |       |       |                                 |
| 51      | June-04       | O141      | B1-1       |      |       |       |       |       |       |       |       |                                 |
| 54      | June-04       | -         | B1-1       |      |       |       |       |       |       |       |       |                                 |
| 9       | March-04      | -         | B1-1       | +    |       |       |       |       |       |       |       |                                 |
| 11      | March-04      | O8        | B1-1       | +    |       |       |       |       |       |       |       |                                 |
| 14      | March-04      | -         | B1-1       | +    |       |       |       |       |       |       |       |                                 |
| 46      | May-04        | -         | B1-1       | +    |       |       |       |       |       |       |       |                                 |
| 43      | May-04        | O8        | B1-1       |      |       |       |       |       |       |       |       |                                 |
| 33      | May-04        | -         | B1-1       | +    |       |       |       |       |       |       |       |                                 |
| 66      | March-05      | O8        | A-2        |      |       |       |       |       |       |       |       |                                 |
| 69      | April-05      | O8        | A-2        |      |       |       |       |       |       |       |       |                                 |
| 75      | June-05       | O8        | B1-1       | +    |       |       |       |       |       |       |       |                                 |
| 74      | June-05       | O8        | B1-1       | +    |       |       |       |       |       |       |       |                                 |
| 19      | April-04      | -         | B1-1       | +    |       |       |       |       |       |       |       |                                 |
| 34      | May-04        | -         | B1-1       | +    |       |       |       |       |       |       |       |                                 |
| 47      | May-04        | -         | B1-1       | +    |       |       |       |       |       |       |       |                                 |
| 48      | June-04       | -         | B1-1       | +    |       |       |       |       |       |       |       |                                 |
| 53      | June-04       | -         | A-1        |      |       |       |       |       |       |       |       |                                 |
| 59      | June-04       | -         | B1-1       |      |       |       |       |       |       |       |       |                                 |
located on MHI813 genome are presented in Table 4. The F17 cluster was otherwise found to be carried by a genomic island, that straddles the contigs 19 and 20 of MHI813 wgs sequences. This genomic island was shown to be inserted in the tRNA pheV gene. Other putative genes were found as well, notably a P4-like integrase gene close to pheV gene, and gene clusters and operons like type VI secretion system gene cluster, microcins I47 and H47 gene cluster (Figure 2), suggesting that it actually constitutes a pathogenicity or fitness island.

The F17e-A and F17-G3 prevalence in healthy bovines in France is very low
To further assess the genetic flux of these two new variants F17e-A and F17-G3, we investigated their prevalence in faecal samples from 787 healthy adult cattle in 6 slaughterhouses in Western France. Among the 787 samples analyzed, 373 were found positive for F17 by qPCR. On the 373 F17-positive samples, 3 were shown to be positive for F17e-A (3/373, 0.8%). No F17-G3 positive sample was detected.

Table 3 Detection and identification of F17 genes variants and virulence-associated genes in the collection of E. coli isolates from feces of calves with diarrhea in Iran (Continued)

| Date   | Month | Year | Hly | Sfi | Lti | Isolates |
|--------|-------|------|-----|-----|-----|----------|
| 62     | March | 05   | B1-1|     |     |          |
| 65     | March | 05   | D-1 |     |     |          |
| 71     | April | 05   | O8  | B1-1|     |          |
| 39     | May   | 04   | O15 | B1-1|     |          |
| 31     | May   | 04   |     | B1-1|     |          |
| 60     | June  | 04   | O49 | B1-1|     |          |
| 70     | April | 05   | A-1 |     |     |          |
| 27     | April | 04   | O8  | B1-1|     |          |
| 20     | April | 04   |     | B1-1|     |          |
| 24     | April | 04   |     | B1-1|     |          |
| 37     | May   | 04   | O8  | B1-1|     |          |
| 41     | May   | 04   | O8  | B1-1|     |          |
| 50     | June  | 04   |     | B1-1|     |          |
| 72     | April | 05   | O15 | B1-1|     |          |
| 55     | June  | 04   | D-1 |     |     |          |
| 61     | June  | 04   | A-2 |     |     |          |

The PFGE profiles of the 20 uncharacterized F17-producing isolates of the collection of E. coli isolates from feces of diarrheic calves in Iran. The PFGE profiles were analyzed with Gelcompar II 6.5 software, using Dice similarity coefficient, 0% optimization, 1% tolerance and UPGMA clustering method. 5 clones were identified, named from clone I to clone V.
Plasmid pVir68 carries a new variant F17-A, which corresponds to a combination of F17c-A and F17d-A. The 6.0900 strain was previously detected positive by PCR with F17-A common primers, and with F17c-A and F17d-A specific primers (data not shown). The 6.0900 strain plasmid pVir68, which was sequenced by Johnson et al. [17], is known to carry a F17 fimbriae encoding gene cluster. To clarify the F17-A status of 6.0900 strain, an in silico analysis of the plasmid pVir68 sequence was undertaken. It showed that the f17A encoding gene on...
pVir68 corresponds to a combination of F17c-A and F17d-A variants, with the N-terminal part corresponding to the same part on f17Ac gene and the C-terminal part corresponding to the same part on f17Ad gene (Figure 3). A 30 bp highly conserved area was also identified between the f17Ac gene corresponding N-terminal part and the f17Ad gene corresponding C-terminal (264–292 bp area on plasmid pVir68 f17A gene). Sequencing of F17c-A specific PCR product obtained on 6.0900 strain was then performed, and the in silico analysis of the genetic sequence of the PCR product confirmed that the f17A gene of pVir68 was a combination of f17Ac and f17Ad genes. We proposed F17f-A for this new variant of the major subunit. f17Af nucleotide sequence is presented in Table 4.

F17e-A and F17-G3 phylogenetically diverge from the other variants of F17-A and F17-G

In order to clarify the phylogenetic links between the 4 F17-A and 2 F17-G variants reported up to now and the F17e-A, F17f-A and F17-G3 newly described, a phylogenetic analysis was performed with the f17A and f17G gene sequences from several E. coli F17-producing strains and E. coli and P. mirabilis F17-related producing strains (Table 1). In the two phenograms, all gene sequences encoding a variant were observed in related leaves (Figure 4). f17Ae gene sequence was found to be related to f17Ab gene sequence. On the other hand, f17G3 gene was found to significantly diverge from the genes coding the two variants F17-G1 and F17-G2.

The FY antiserum detects F17a-A variant-producing isolates only

The FY antiserum is commonly used in veterinary laboratories to detect all F17-producing E. coli isolates involved in diarrhea or septicemia outbreak in calves. To check the ability of this antiserum to detect all F17-A variants, we performed agglutination testing using the appropriate reference strains for each F17-A variant, including the MHI813 and 6.0900 strains for the new variants F17e-A and F17f-A. A positive agglutination reaction was only observed with the 25KH9 reference strain only, suggesting that this antiserum is unable to detect other F17a-A variants.

Discussion

In this study, we report three new variants of the pilin and adhesin subunits F17A and F17G of the F17 fimbriae in E. coli. The F17e-A variant was identified in E. coli isolates recovered from feces of diarrheic calves in Southeastern Iran. The prevalence of this variant among the F17-A positive E. coli isolates (71.4%) was surprisingly high in this cattle population. These F17e-A isolates were identified in 7/11 herds, suggesting a wide spread of the variant F17e-A in the Kerman province. These isolates were found in 5 E. coli clones, three of them harbouring the F17e-A variant only. Moreover, the cnf2 and cdtIII genes were found in association with F17e-A in a fourth clone. Interestingly, the most prevalent clone I and the 3 clones II, III and V were detected in the same herds suggesting the presence of a major clone disseminating the F17e-A variant. Considering that a plasmidic location of the f17Ae gene would probably be associated with a higher clonal diversity of the f17Ae-carrying E. coli thanks to intercellular plasmid spread, the hypothesis of a chromosomal location of f17Ae can be put forward. However, this would need further molecular confirmation.

In three clones (I, II and IV), the F17e-A variant was the unique virulence-associated factor identified. Considering that those calves were severely affected with diarrhea, this may suggest the expression of a still unknown virulence factor associated to the F17e-A variant. Therefore, it could be of interesting value to further determine the whole sequence genome of these E. coli clones in order to detect in silico possible new virulence factors involved in diarrhea outbreak in calves.

The new variant F17e-A was also identified in the E. coli MHI813 strain, which was isolated from bovine feces. It was found to be associated on the MHI813 strain genome to a new variant of the adhesin subunit, F17-G3. These two variants were found into a gene cluster, confirming the previous observations of a co-localization of the F17 fimbriae encoding genes in a cluster organization [10,35]. Moreover, the co-localization of a type VI secretion gene cluster, and a microcin I47 and H47 gene cluster is reminiscent of a pathogenicity or fitness island [36,37]. The integration of this genomic island within the pheV gene, which encodes phenylalanine
tRNA, also supports this hypothesis. Indeed, the pheV gene is localized at the 67th minute on the E. coli K12 genome map, which is a known hot spot for pathogenicity island insertions [38-41].

The new variant F17-G3 was found to share no identity on its N-terminal encoding part with the two already reported variants F17-G1 and F17-G2, what was highlighted by the great phylogenetic divergence of F17-G3 with the two others adhesin variants. The N-terminal part of the adhesin is known to be responsible for the adhesion onto the host receptor [4] and the completely different N-term part of F17-G3 may indicate some differences in receptor specificity or even in host tropism. Therefore, a functional characterization of this new variant may help investigating the F17-G3 adhesion properties. Concerning the two variants F17-G1 and F17-G2, the phylogenetic analysis showed a very close relatedness. These two variants share 99% identity, and we can wonder whether the weak genetic differences between these two variants do really correspond to biological differences, e.g. in terms of adhesion specificity.

Regarding the genetic flux of the two new variants F17e-A and F17-G3, the prevalence of the variant F17e-A was very low in healthy adult bovines in France. F17-
G3 was even not detected at all in these animals. One hypothesis would rely on a very low prevalence in cattle, those variants being incidentally identified in a restricted area (Iran) during a short period of time. Another hypothesis could be that these two variants are strongly associated with virulence in calves, as it is the case for the F17b-A variant, which is known to be mainly associated to CNF2 producing \textit{E. coli} strains, both of them being carried by the \textit{Vir} plasmid [4,10,14].

Another new variant F17f-A of the major subunit was identified on the recently sequenced virulence plasmid pVir68, that carries different virulence factors encoding genes, like \textit{cnf2} and \textit{cdtIII} genes [17]. This new variant corresponds to a combination of the already described variants F17c-A and F17d-A. The structure of this new variant suggests that the highly conserved regions previously identified [4] on the different variants of the pilin and adhesin subunits could be the target of recombination events, which would constitute a potential source of genetic diversity. This strategy may allow the F17-producing strains to bypass the immune defense system of the host, particularly the specific antibodies developed producing strains to bypass the immune defense system of genetic diversity. This strategy may allow the F17-encoding genes on the isolates from the collection. MB detected F17 encoding genes on the isolates from the collection. MB detected F17 encoding genes on the isolates from the collection.

EO and JYM conceived the study. RG collected the feces samples from diarrheic calves in Iran, performed the PFGE on the F17e-A-positive isolates, performed the in silico analyses on \textit{E. coli} MBI813 genome and pVir68 sequence, the phylogenetic analyses of F17-A and F17-G variants, designed the specific primers for F17e-A and F17-G3, detected these two variants in the French bovines collection and assessed FY antiseraum detection. LC, PC provided support for PFGE. MB provided technical support. HB provided the French bovines feces collection. MB interpreted all the results and wrote the manuscript. All authors read and approved the manuscript.

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