Genomic and phenotypic characterization of *Escherichia coli* isolates recovered from the uterus of puerperal dairy cows

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ABSTRACT

The role of *Escherichia coli* in the pathogenesis of the puerperal uterine infection of the cow is largely unknown. It is proposed that *E. coli* favors the persistence of *Arcanobacterium pyogenes* and gram-negative bacteria that are pivotal to the establishment of the infection. Here, we report the genomic and phenotypic characteristics of 72 *E. coli* isolates recovered from the uterus of dairy cows with normal puerperium (n = 12; 35 isolates) or clinical metritis (n = 18; 37 isolates), in an attempt to identify characteristics that are related to the establishment of uterine infection. We evaluated DNA fingerprints generated by repetitive element sequence-based PCR, phylogenetic grouping, the presence of 15 virulence factor genes, in vitro biofilm formation and its relationship to curli fimbriae expression, and cellulose production. We found a wide genetic diversity (40 clonal types), including types common to normal puerperium and clinical metritis cows (n = 6), as well as types specific to normal puerperium (n = 14) or clinical metritis (n = 20) cows. Isolates were assigned to phylogenetic groups B1 (58%), A (31%), and D (11%). Only 4 virulence factor genes were detected (*hlyE*, *hlyA*, *iuc*, and *eaeA*). In vitro biofilm formation was significantly affected by culture medium and incubation temperature. Curli fimbriae expression and cellulose production, although related to biofilm formation, were not required for it. None of the evaluated *E. coli* characteristics were significantly related to the establishment of the uterine infection. In conclusion, data presented in this paper indicate that *E. coli* isolates recovered from the uterus of puerperal cows present a wide genetic diversity, do not belong to a known pathogenic group, and have a low potential of virulence and persistence. This corroborates the putative role of the bacterium in the pathogenesis of the puerperal uterine infection of the cow.

Key words: *Escherichia coli*, virulence factor gene, biofilm, puerperal cow

INTRODUCTION

The role of *Escherichia coli* in the pathogenesis of the puerperal uterine infection of the cow is unclear. It has been implicated in early ovarian disturbances and appears to increase the susceptibility of the uterus to subsequent infections with *Arcanobacterium pyogenes* and gram-negative anaerobes, the former being relevant to the establishment and persistence of uterine infection (Miller et al., 2007; Williams et al., 2008). Although *E. coli* is the most prevalent bacterium isolated from the uterus during the first week postpartum, infection will only be established in a small subset of cows (Mateus et al., 2002a). Establishment of the infection is probably dependent on the host defense mechanisms, type and virulence of the bacteria involved (Silva et al., 2008), and environmental conditions. To what extent the genetic and phenotypic characteristics of *E. coli* uterine isolates are related to the establishment of the puerperal uterine infection in the cow is largely unknown.

Phylogenetic analysis has been used to evaluate the evolutionary origins of pathogenic *E. coli* strains. Four main phylogenetic groups were described: A, B1, B2, and D (Herzer et al., 1990). Extraintestinal pathogenic *E. coli* strains belong mainly to group B2 and harbor several virulence factor (VF) genes and, in a few cases, to group D (Picard et al., 1999; Johnson and Stell, 2000). In contrast, most commensal *E. coli* strains belong to groups A and B1 and harbor few VF genes compared with the corresponding pathogenic strains (Duriez et al., 2001). The relationship between the presence of putative VF genes in *E. coli* and the establishment of the puerperal uterine infection in the cow was not reported.

The evaluation of the genetic diversity between strains allows the identification of clonal types that may be associated with the disease. In *E. coli* strains this evaluation is better achieved by 2 techniques of the repetitive element sequence-based PCR (rep-PCR) typing

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method (Versalovic et al., 1991). These techniques use primers directed to the enterobacterial repetitive intergenic consensus (**ERIC**; Hulton et al., 1991) and to the repetitive extragenic palindromic (**REP**) consensus (Stern et al., 1984).

The ability of some *E. coli* strains to form biofilm may promote their persistence and tolerance to antimicrobial agents. In *E. coli*, biofilm formation is associated with the expression of curli fimbriae, production of exopolysaccharides such as cellulose, and expression of type I and conjugative pili and flagella (Van Houdt and Michiels, 2005). Curli fimbriae enhance bacterial adherence to mammalian host cells (Wang et al., 2006), mediate invasion of epithelial cells (Gophna et al., 2001), and trigger an immediate immune response (Wang et al., 2006). Expression of curli fimbriae is regulated through environmental and genetic factors. The genes for curli fimbriae production are organized in the operons *csgBA* (C) and *csgDEFG* but only some of these genes have fully identified functions. The *csgBA* operon encodes the major structural subunit, CsgA, and a minor subunit, CsgB, a surface-exposed nucleator (Hammar et al., 1995); CsgD is a transcriptional regulator belonging to the LuxR superfamily and is required for the activation of curli fimbriae as well as cellulose biosynthesis (Hammar et al., 1995). The exopolysaccharide cellulose is frequently co-produced with curli fimbriae by commensal *E. coli* strains. A typical commensal isolate expresses curli fimbriae and produces cellulose at 28°C and 37°C (Bokranz et al., 2005).

The objective of this study was to characterize *E. coli* isolates recovered from the uterus of cows with either normal puerperium or clinical metritis, to identify genomic and phenotypic characteristics associated with the uterine infection. The genomic characterization included the identification of VF genes by PCR, phylogenetic grouping, and evaluation of the genetic diversity. The phenotypic characterization included in vitro biofilm formation and its relationship with curli fimbriae expression and cellulose production.

MATERIALS AND METHODS

Animals and Bacterial Isolates

The *E. coli* isolates ($n = 72$) were recovered from uterine swabs taken during the puerperium of Holstein dairy cows from herds I ($n = 17$) and II ($n = 13$). Cows were clinically monitored twice a week and uterine swabs taken once a week from parturition until 2 consecutive negative cultural samples were obtained or until 46 d postpartum, whichever occurred first. Cows presented either a normal puerperium ($n = 12$; 35 isolates) or clinical metritis ($n = 18$; 37 isolates). The methodologies

of swabbing, bacterial isolation, and species identification were described previously (Mateus et al., 2002a,b; Silva et al., 2008). Briefly, after swabbing the uterus through a sterile procedure, swabs were inoculated into Columbia 5% sheep blood agar (bioMérieux, Marcy L'Etoile, France) and MacConkey agar (Merck, Darmstadt, Germany) plates and were incubated at 37°C overnight. Colonies with phenotypic characteristics of *E. coli* and that were lactose fermenting on MacConkey agar were selected for identification with the API 20E system (bioMérieux). Upon identification, the isolates were kept frozen at -80°C . Hemolytic phenotypes were detected in sheep and horse blood agar plates. Reference strains *E. coli* J96, KS52, CECT 685, CECT 4782, ATCC 43895 (O157:H7), Utrecht 1309, and Utrecht 805 were used as positive controls for virulence factors and genotyping. The reference strain *E. coli* 25922 was used as a negative control for curli fimbriae expression and cellulose production. *Candida albicans* was used as a positive control for cellulose production. In each plate, 2 *E. coli* strains isolated in this work (I.6-R23–17; II.14-R28–6) were used as positive controls for curli fimbriae expression and cellulose production.

Genomic DNA Isolation

Isolates of *E. coli* and reference strains were grown on Columbia 5% sheep blood agar plates (bioMérieux) at 37°C for 16 h. The DNA was extracted from approximately one loop of biomass using the EasySpin kit (Citomed, Lisbon, Portugal), the concentration and purity were evaluated by optical density (at 230, 260, and 280 nm), and integrity was assessed through a 0.8% agarose gel electrophoresis.

Clonal Analysis

Genomic DNA fingerprinting was generated by ERIC and REP-PCR (Versalovic et al., 1991). ERIC-PCR was performed with ERIC2 primer (5' AAGTAAGTGACTGGGGTGAGCG 3') and REP-PCR with 2 opposing primers, REP1R-I (5' IIIICGICGICATCIGGC 3') and REP2-I (5' ICGICTTATCIGGCCTAC 3'). The reactions occurred in a thermal cycler (Bio-Rad Laboratories Inc., Hercules, CA) in a 25- μL volume reaction mixture containing 2 μM of each primer, 100 ng of genomic DNA, 0.1 mM of each deoxynucleotide triphosphate (Promega, Madison, WI), 1 $\times$ PCR buffer, 2 mM of MgCl_2 , and 2 U of GoTaq Flexi DNA polymerase (Promega). An initial denaturation step (95°C, 7 min) was followed by 30 cycles of denaturation (90°C, 30 s), annealing (ERIC: 52°C, 1 min; REP: 40°C, 1 min) and extension (72°C, 8 min), with a single final extension cycle (72°C, 16 min). A negative control consisting of

the same PCR mixture without DNA was included in all PCR sessions. The PCR products (5 μ L for ERIC and 8 μ L for REP) were run through an agarose gel (ERIC: 1.5%; REP: 1%) containing ethidium bromide and, after 4 h at 70 V, the gels were photographed with an ImageMaster VDS System (Amersham Pharmacia Biotech, Oeiras, Portugal). Band sizes were determined by comparison with a standard DNA ladder (1 Kb Plus DNA Ladder, Invitrogen, Lisbon, Portugal). The DNA fingerprints were analyzed in Bionumerics version 4.61 from Applied Maths (Sint-Martens-Latem, Belgium).

The degree of similarity between fingerprints was calculated with the Pearson product moment correlation coefficient and clustering was based on the unweighted pair group method using arithmetic averages (UPGMA). Three dendrograms were assembled, one for each technique of rep-PCR (ERIC and REP) and the third for the composite data (ERIC-plus-REP). The reproducibility of ERIC and REP was evaluated by comparing the amplification products of 2 different PCR runs for 12 isolates, and the average reproducibility for each rep-PCR technique was used to set up the similarity cut-off level for type identification. On the composite data, a type was defined based on the average reproducibility of ERIC-plus-REP fingerprints. The discriminatory power of each rep-PCR technique was calculated by Simpson's numerical index of diversity (**SID**; Hunter and Gaston, 1988), and the 95% confidence interval (95% CI) was calculated according to Grundmann et al. (2001). This index calculates the probability of 2 unrelated isolates sampled randomly from the population being placed into different types. The agreement between the typing techniques (ERIC, REP, ERIC-plus-REP) was evaluated by the adjusted Rand (**AR**) coefficient (Carriço et al., 2006; <http://www.comparingpartitions.info/>). The greater the AR value (scale 0–1), the higher the level of agreement between methods.

Phylogenetic Grouping

Phylogenetic grouping was performed using a triplex PCR targeting the genes *chuA* and *yjaA*, and the DNA fragment TspE4-C2 as described by Clermont et al. (2000). This method allows the discrimination of 4 phylogenetic groups: A, B1, B2, and D. According to this method, isolates negative for *chuA* and TspE4-C2 were assigned to group A, isolates negative for *chuA* and positive for TspE4-C2 were assigned to group B1, isolates positive for *chuA* and *yjaA* were assigned to group B2, and isolates positive for *chuA* and negative for *yjaA* were assigned to group D. *Escherichia coli* J96 and verotoxin-producing *E. coli* O157:H7 (ATCC 43895) were used as positive controls for phylogenetic groups B2 and D, respectively.

Detection of VF

Conventional PCR was used to detect the presence of 15 *E. coli* VF genes: *papEF* (P-fimbriae), *sfaDE* (S-fimbriae), *afaBC* (afimbrial adhesion 1 or Afa1), *hlyA* (α -hemolysin), *cnf1* (cytotoxic necrotizing factor 1, CNF1), and *iucD* (aerobactin) were tested using a multiplex described by Yamamoto et al. (1995); *cnf2* (cytotoxic necrotizing factor 2, CNF2) was tested according to procedures described by Kaipainen et al., (2002); *sta* (heat-stable enterotoxin a), *stx1* and *stx2* (shiga toxins 1 and 2), *eaeA* (intimin), F5 (K99) and F41 fimbriae were detected by an optimized multiplex using the primers described by Franck et al. (1998) and the conditions described by Güler and Gündüz (2007); F17 fimbriae was detected using a multiplex with 3 primers (P7, P8, and P9) according to Güler and Gündüz (2007); *hlyE* (hemolysin E) primers (Fw-5' GAAACCGCAGATG-GAGCATT 3'; Rv-5' CGCCCGCAGCAATAGAATAG 3') were designed with Primer3 software (<http://fokker.wi.mit.edu/primer3>; Rozen and Skaletsky, 2000) using the gene sequence deposited in GenBank (accession no. NC_000913).

Genes for the curli subunit protein, *csgA*, and the curli transcriptional regulator, *csgD*, were targeted and amplified by conventional PCR. Sequences deposited in the GenBank (accession no. NC_X90754) were used for *csgA* and *csgD* primer design, using Primer3 software (*csgA*: Fw-5' CAGCAATCGTATTCTCCGGTA 3'; Rv-5' CGTTGTTACCAAAGCCAACC 3' and *csgD*: Fw-5' TTATCGCCTGAGGTTATCGTTT 3'; Rv-5' TAAATCTTCTTTGCAGGCGACA 3').

The PCR was performed in a 25- μ L volume reaction mixture containing 25 pmol of each primer, 0.1 mM of each deoxynucleotide triphosphate (Promega), 1 $\times$ PCR buffer, 2 mM MgCl₂, 200 ng of genomic DNA, and 2 U of GoTaq Flexi DNA polymerase (Promega). The amplification conditions included an initial denaturation step at 94°C (5 min), followed by 25 cycles of denaturation at 94°C (2 min), annealing at 58°C (1 min) and extension at 72°C (1 min), with a single final extension cycle at 72°C (3 min). A negative control consisting of the same PCR mixture but without DNA was included in all PCR sessions. The identity of the PCR products was initially confirmed by DNA sequencing. Amplification products were separated by electrophoresis through a 1.5 or 1% agarose gel containing ethidium bromide, and the bands were visualized with the ImageMaster VDS System (Amersham Pharmacia Biotech). The result was considered positive if the amplification product had the expected molecular size. Strains *E. coli* J96 (positive for *hlyA*, *cnf1*, *sfa*, and *pap*), KS52 (positive for *afa*, *iuc*, and *pap*), CECT 4782 (positive for *stx1*, *stx2*, and *eae*), CECT 685 (positive for *sta*), ATCC 43895 (O157:H7;

positive for *hlyE*), Utrecht 1309 (0101:F41; positive for F41), and Utrecht 805 (positive for F5) were used as positive controls in the PCR reactions.

Detection of Curli Fimbriae Expression

Isolates grown overnight in Luria Bertani (LB) broth medium at 37°C were spotted onto Congo red indicator (CRI) agar made with YESCA agar supplemented with 20 mg/L of Congo red (C6767, Sigma, St. Louis, MO) and 10 mg/L of Coomassie Brilliant Blue G dye (B0770, Sigma). The CRI plates were incubated at 37°C for 24 h and at 28°C for 48 h (Bokranz et al., 2005). *Escherichia coli* expressing curli fimbriae bind to Congo red dye and form red colonies, whereas curli-negative bacteria form smooth, white colonies.

Detection of Cellulose

Cellulose was visualized on YESCA agar plates containing 5 mL/L of calcofluor (calcofluor white stain, Fluka, Sigma-Aldrich Chemie GmbH, Steinheim, Germany), which detects the β -1,4 glucose linkages of cellulose. Fluorescent colonies were observed under a 366 nm UV light source after incubation for 24 h at 37°C or 48 h at 28°C (Bokranz et al., 2005). Faint fluorescence was regarded as negative.

In Vitro Biofilm Formation Assays

Biofilm formation assays were performed following a described previously method (Naves et al., 2008) with minor modifications. Briefly, overnight liquid LB cultures without salt (NaCl) were diluted to reach a standardized optical density (OD)_{640nm} of 0.2, and 20 μ L of this cell suspension was used to inoculate 4 replicate wells of a 96-well cell-treated polystyrene microtiter plate (Greiner GmbH, Frickenhausen, Germany) containing 180 μ L of M9 or LB medium without NaCl. Wells containing sterile medium were used as negative controls. Plates were incubated at 37°C for 24 h or at 28°C for 48 h without agitation. Bacterial growth was assessed by measuring the OD_{620nm} of the cell suspensions with a microplate OD reader (SpectraMax 340 Pc, Decada Bio, Lisbon, Portugal). For biofilm quantification, broth was removed and the wells were rinsed 2 times with 200 μ L of PBS. Adherent bacteria were stained with a 1% crystal violet solution for 15 min at room temperature. After 3 more rinses with 200 μ L of water, the plates were air-dried for 1 h, and the dye associated with the adherent cells was solubilized in 200 μ L of absolute ethanol. The OD_{540nm} of this solution was then measured in the OD microplate reader.

The amount of biofilm formation was determined by applying the following formula (Niu and Gilbert, 2004): $SBF = (AB - CW)/G$, where SBF is the specific biofilm formation index, AB is the OD_{540nm} of stained attached bacteria, CW is the OD_{540nm} of stained control wells containing bacteria-free medium, and G is the OD_{620nm} of the initial cell suspension. Biofilm formation was classified semiquantitatively as positive (SBF ≥ 0.70) or negative (SBF < 0.69), based on Naves et al. (2008) classification.

Statistical Analysis

Data were analyzed using SAS software (version 9.1; SAS Institute Inc., Cary, NC). Categorical data were analyzed by chi-square test or Fisher's exact test when required. The SBF values were analyzed by multivariate ANOVA (MANOVA), considering 3 main effects: culture medium (LB vs. M9), incubation temperature (37°C vs. 28°C), and type of puerperium (normal vs. clinical metritis). Biofilm formation, curli fimbriae expression, and cellulose production were also analyzed by MANOVA, after text value transformation, considering these 3 main effects for biofilm formation and the temperature and type of puerperium for curli expression and cellulose production.

Effects were further analyzed by the Scheffé test for a minimum significance level of 5% ($P < 0.05$). Categorical data on biofilm formation were also analyzed by Fisher's exact test considering the effects of culture medium and the incubation temperature and the incubation temperature and the type of puerperium.

RESULTS

Comparison Between REP-PCR and ERIC-PCR Fingerprinting Techniques

According to the average reproducibility (96 ± 3.9 for ERIC, 95 ± 4 for REP, and 95 ± 3.1 for ERIC-plus-REP), the cut-off levels for type identification were set at 92% for ERIC, 91% for REP, and 92% for ERIC-plus-REP. Based on these cut-off levels, the highest discriminatory power (evaluated by the SID) was obtained by the ERIC-plus-REP technique, but for the 3 methods the discriminatory power was $\geq 94\%$ (Table 1). In general, the patterns of REP fingerprints were more complex than those of ERIC (Figure 1). All *E. coli* isolates were typed by both techniques. The number of types identified by each of the techniques, the most prevalent types, and the number of types represented by a single isolate are presented in Table 1.

The agreement between ERIC, REP, and ERIC-plus-REP results (evaluated by the AR coefficient) was

Table 1. Data generated by the repetitive element sequence-PCR (rep-PCR) typing techniques including the 72 *Escherichia coli* uterine isolates and 5 reference strains¹

rep-PCR ²	Clonal types, n	Isolates of most prevalent type, n	Single types, n (%)	Simpson's index of diversity (95% CI)
ERIC	26	13	9 (11.69)	94.22 (91.88–96.57)
REP	32	9	19 (24.68)	95.39 (93.71–97.07)
ERIC-plus-REP	44	8	30 (38.96)	97.33 (95.96–98.71)

¹Clonal types = number of clonal types generated by the different rep-PCR typing techniques; single types = number (%) of clonal types with only one strain generated by the different rep-PCR typing techniques; 95% CI = 95% confidence interval.

²ERIC = enterobacterial repetitive intergenic consensus; REP = repetitive extragenic palindromic consensus.

the highest between REP and ERIC-plus-REP (AR = 0.7170; i.e., 72% agreement). The AR values for ERIC-PCR and REP-PCR and for ERIC and ERIC-plus-REP agreements were 0.2973 and 0.3457, respectively. Therefore, the clonal type relationships and the following analysis were conducted based on the dendrogram obtained through the ERIC-plus-REP-PCR fingerprints (Figure 1).

Cluster Analysis of ERIC-Plus-REP-PCR Genomic Fingerprints

From the 72 isolates, 40 fingerprint patterns were identified (including one shared by a uterine isolate and the reference strain KS52, Figure 1). Another 4 fingerprint patterns were identified in the other reference strains. Herd I showed more genetic variability than herd II: 24 types within 35 isolates (69%) versus 17 types within 37 isolates (46%), respectively. All types were herd-specific (except type 1 that was isolated in 2 cows from herd II and in 1 cow from herd I; Figure 1; Table 2). In 50% of the cows, 2 to 3 different types were identified throughout the puerperium. Twenty types were identified only in cows that developed clinical metritis, whereas 14 types were identified only in cows with normal puerperium. However, these types were represented either by one isolate or by multiple isolates from the same animal. The remaining 6 types were identified in cows with normal puerperium or clinical metritis (Table 3).

Phylogenetic Grouping

Escherichia coli isolates were assigned to phylogenetic group B1 (57.7%), group A (30.9%), and group D (11.3%). No isolates were assigned to group B2. All isolates from the same type were assigned to the same phylogenetic group (Figure 1). The 3 phylogenetic groups were equally present in normal puerperium and clinical metritis cows (Table 2, 3). However, considering together the groups clinical metritis and the composite

(normal puerperium + clinical metritis; both, Table 3), phylogenetic group A was significantly more represented in the composite group than in the normal puerperium group ($P = 0.05$). In contrast, phylogenetic group D was significantly ($P < 0.05$) more represented in the normal puerperium group than in the composite group. We were not able to assign a phylogenetic group to reference strain CECT 685 and to its closely related bovine isolate (I.6).

Detection of VF Genes

Data regarding the detection of VF genes are summarized in Tables 2 and 3. Of the 15 VF genes tested, only 4 (*hlyE*, *hlyA*, *iuc* and *eaeA*) were detected in the genome of *E. coli* bovine isolates. All isolates exhibited hemolysis on horse blood agar and carried the gene *hlyE*; 8 of these isolates were also positive for the *hlyA* gene and exhibited α -hemolysis on sheep blood agar. The *hlyA*-positive isolates were all collected from herd I cows. The *iucD* gene was detected in 30 (41.7%) isolates, but the prevalence of this gene was different in the 2 herds [5 of 35 (14%) isolates of herd I vs. 25 of 37 (68%) isolates from herd II; $P < 0.01$]. Only one isolate harbored the *eaeA* gene, and none of the other genes was detected. Reference strain J96 exhibited α -hemolysis on sheep and horse blood agar and carried the *hlyA* gene but not the *hlyE* gene. In contrast, reference strains KS52, CECT 685, and 4782 exhibited hemolysis only on horse blood agar and carried the *hlyE* gene but not the *hlyA* gene. There was no relationship between the presence of uterine infection and the presence of VF genes in the isolates (Table 3).

Phenotypic Curli Fimbriae Expression and Cellulose Production

The MANOVA showed that curli fimbriae expression was not significantly affected by the type of puerperium, the incubation temperature, and their interaction. Cellulose production was only significantly affected (P

Figure 1. Dendrogram generated by Bionumerics (Applied Maths, Sint-Martens-Latem, Belgium) software of composite (ERIC-plus-REP-PCR; ERIC = enterobacterial repetitive intergenic consensus; REP = repetitive extragenic palindromic consensus) fingerprints of 72 uterine bovine *Escherichia coli* isolates and 5 reference strains (CECT4782, O157, CECT685, KS52, and J96). The degree of similarity (%) between fingerprints is given at the top by the Pearson coefficient. The clonal types were defined at the cut-off level of 92%. PG = phylogenetic group; Status: N = normal puerperium, M = clinical metritis; DPP = days postpartum of isolation; NG = without phylogenetic group identification.

Table 2. Relationship between clonal types, phylogenetic groups and the presence of virulence factor genes in 72 uterine bovine *Escherichia coli* isolates¹

Clonal type reference	Puerperium	Isolates	Herd	PG	Virulence factor gene ²		
					<i>hlyA</i>	<i>hlyE</i>	<i>iuc</i>
1	N; M	8	I; II	B1	1/8	8/8	7/8
16	N; M	6	II	A	0/6	6/6	6/6
39	M	5	II	A	0/5	5/5	5/5
12	N; M	4	II	B1	0/4	4/4	0/4
32	N; M	4	I	A	4/4	4/4	0/4
35	N	3	I	D	0/3	3/3	0/3
18	N	3	I	B1	0/3	3/3	0/3
28	N	2	II	D	0/2	2/2	2/2
17	N; M	2	II	A	0/2	2/2	2/2
34	M	2	I	A	1/2	2/2	0/2
41	N	2	I	A	0/2	2/2	2/2
2; 24	N; M	2	I	B1	0/4	4/4	0/4
8	N	2	I	B1	0/2	2/2	2/2
4; 5; 6; 13; 14; 15; 22; 23; 25; 26	N; M	1	I	B1	0/10	10/10	0/10
7; 9; 10; 11; 19; 20; 29	N; M	1	II	B1	0/7	7/7	0/7
3; 27	N; M	1	I	B1	2/2	2/2	0/2
21	N	1	I	B1	0/1	1/1	1/1
36	M	1	I	NG	0/1	1/1	0/1
38	M	1	II	D	0/1	1/1	0/1
40	M	1	II	A	0/1	1/1	1/1
43; 44	M	1	II	D	0/2	2/2	2/2

¹Puerperium: N = normal (n = 12, 35 isolates); M = clinical metritis (n = 18, 37 isolates); PG = phylogenetic group; NG = without phylogenetic group identification.

²Number of isolates expressing the virulence factor gene/total number of isolates. *hlyA* = α -hemolysin; *hlyE* = hemolysin E; *iuc* = aerobactin.

< 0.00001) by the incubation temperature: incubation at 28°C resulted in significantly higher cellulose production than incubation at 37°C. Categorical data analysis of curli fimbriae expression and cellulose production gave similar results to those obtained by MANOVA; these results are presented in Table 4.

Whenever more than one isolate was obtained from the same swab, the isolates displayed the same phenotype. At both incubation temperatures, all cellulose-negative isolates were also curli fimbriae-negative, and all cellulose-positive isolates were also curli fimbriae-

positive. The curli subunit gene, *csgA*, and the curli transcriptional regulator gene, *csgD*, were both present in all isolates. Curli- and cellulose-producing *E. coli* strains did not selectively cluster when fingerprinted by rep-PCR.

Biofilm Formation

The MANOVA showed that SBF values were significantly affected by culture medium ($P < 0.0001$) and by incubation temperature ($P < 0.05$), but not by the

Table 3. Relationship between type of puerperium and genetic characteristics of *Escherichia coli* isolates

Puerperium (clonal types)	Phylogenetic group	Number of isolates (n = 72)	Virulence factor genes, ¹ n		
			<i>hlyA</i>	<i>hlyE</i>	<i>iuc</i>
Normal (n = 14)	A	2	0	2	2
	B1	13	1	13	3
	D	5	0	5	2
Clinical metritis (n = 20)	A	8	1	8	6
	B1	14	1	14	0
	D	3	0	3	2
	NG ²	1	0	1	0
Both ³ (n = 6)	A	12	4	12	8
	B1	14	1	14	7
	D	0	0	0	0

¹*hlyA* = α -hemolysin; *hlyE* = hemolysin E; *iuc* = aerobactin.

²NG = without phylogenetic group identification.

³Both = includes clonal types with strains of normal and clinical metritis puerperium groups.

Table 4. Effects of incubation temperature and culture medium on in vitro biofilm formation as associated curli fimbriae expression and cellulose production of *Escherichia coli* isolates

Phenotype (n = 72 isolates)	Incubation temperature	
	37°C, n (%)	28°C, n (%)
Curli fimbriae expression	56 (77.7)	60 (83.3)
Cellulose production	18 (25.0) ^a	38 (52.8) ^b
Curli fimbriae plus cellulose	18 (25.0) ^a	38 (52.8) ^b
Biofilm formation in Luria Bertani medium	66 (91.6) ^x	71 (98.6) ^x
Biofilm formation in M9 medium	40 (55.5) ^y	40 (55.5) ^y

^{a,b,x,y}Categorical data were analyzed by chi-square test: ^{ab} (for columns), ^{xy} (for rows): $P < 0.05$.

type of puerperium ($P = 0.76$) or by any interaction of the main effects. Incubation at 28°C and culture in LB medium yielded significantly higher SBF values, more indicative of biofilm formation, than incubation at 37°C and culture in M9 medium. In general, SBF values were present in an increasing pattern in the following combinations: M9 at 37°C < M9 at 28°C ≤ LB at 37°C < LB at 28°C. At both incubation temperatures, biofilm formation occurred in significantly more isolates cultured in a rich medium without salt (LB) than in a minimal nutrient medium (M9; $P < 0.05$). Tables 4 and 5 present the above main effects on biofilm production, after categorical data analysis, which yielded similar results to those obtained by MANOVA.

Biofilm formation was not significantly associated with curli fimbriae expression and cellulose production for *E. coli* isolates cultured in LB medium at either incubation temperature (data not shown). In contrast, the above relationship tended to be significant ($P < 0.10$) or reached significance $P < 0.05$ for isolates cultured in M9 medium at both incubation temperatures (data not shown). A relationship between biofilm formation and development of uterine infection was not observed (Table 5).

DISCUSSION

In this study, the genetic and phenotypic characteristics of cow uterine *E. coli* isolates were evaluated in an attempt to characterize the role of this bacterium in the pathogenesis of puerperal uterine infection of the cow.

To assess the genomic diversity among the *E. coli* isolates, 2 rep-PCR techniques (ERIC and REP) were compared. The agreement between the clusters defined by ERIC and REP was only about 30% although the discriminatory power and reproducibility of both techniques were similarly high (>94%). This indicates that these typing techniques are not redundant as also reported by others (McLellan et al., 2003). The REP typing provided more information than ERIC typing, which may be related to the use of 2 primers in the REP technique and to the different numbers of REP and ERIC sequences in the *E. coli* genome. The combination of ERIC and REP typing provided more information and greater discriminatory power than either of the techniques alone and was the approach selected to evaluate the genetic diversity among the *E. coli* isolates.

High genomic diversity was found among the *E. coli* isolates. This genetic heterogeneity was present within both herds and all but one clonal type were herd specific. Although horizontal DNA transfer and mutations cannot be ruled out (Paulin-Curlee et al., 2007), we hypothesize that this genetic diversity might be associated with genome size differences detected by rep-PCR, as reported by Bergthorsson and Ochman (1998). The observation that the same clonal type was recovered from the uterus of different cows is consistent with the hypothesis of *E. coli* being transmitted between animals. Although normal puerperium- and clinical metritis-specific clonal types were identified, the more prevalent types were present both in cows with normal puerperium and clinical metritis. Therefore, a relation-

Table 5. Relationship between establishment of uterine infection and in vitro biofilm formation capability of *Escherichia coli* isolates¹

Type of puerperium ²	Number of isolates	Number (frequency, %) of biofilm formation			
		Rich, 37°C	Rich, 28°C	Minimal, 37°C	Minimal, 28°C
Normal (n = 12)	35	31 (89)	34 (97)	19 (54)	18 (51)
Clinical metritis (n = 18)	37	35 (95)	37 (100)	21 (57)	22 (59)

¹Rich = Luria Bertani (LB) medium without NaCl; minimal = M9 medium.

²No significant differences on biofilm formation were found between the 2 types of puerperium.

ship between clonal type and presence of uterine infection cannot be deduced from our data.

Phylogenetic grouping allocated isolates mainly to groups A and B1 and, to a lesser extent, to group D. No isolate was allocated to group B2. Among extraintestinal pathogenic *E. coli*, group B2 is more frequent and potentially more pathogenic—strains allocated to this group harbor several VF genes (Picard et al., 1999). However, extraintestinal virulence genes were not detected among uterine *E. coli* isolates. Intestinal commensal strains belong to groups A and B1 and carry a small number of virulence determinants (Duriez et al., 2001). Although the majority of diarrheagenic *E. coli* belong to the phylogenetic groups A and B1, typical virulence-associated genes of diarrheagenic *E. coli* were not detected (with one exception) in the 72 isolates. The phylogenetic grouping presented here and the detection of a small number ($n = 4$) of VF genes indicate that bovine uterine *E. coli* isolates possess low virulence potential.

The VF gene *hlyE* was present in all isolates. This gene codes for HlyE, a cytolytic pore-forming toxin unrelated to HlyA, a pore-forming hemolysin of the RTX family (Oscarsson et al., 1999). Eight isolates also carried the *hlyA* gene. Kerényi et al. (2005) suggested that because of interference between the mechanisms regulating production of HlyA and HlyE, the coexistence of *hlyA* and *hlyE* in the same *E. coli* chromosome is probably incompatible. However, corroborating our finding, both genes were detected in uropathogenic *E. coli* strains (including J96) and enteroaggregative *E. coli* strains (Bruant et al., 2006). Expression of HlyE in the absence of the RTX toxins is sufficient to give a hemolytic phenotype in *E. coli* (Ralph et al., 1998). However, *E. coli* isolates carrying the *hlyA* gene produced α -hemolysis in horse and sheep blood agar plates, whereas *E. coli* isolates carrying the *hlyE* gene displayed a hemolytic phenotype only on horse blood agar plates. We did not evaluate uterine *E. coli hlyE* gene expression during the course of infection and therefore we cannot deduce a role for HlyE in the establishment of infection. However, it is possible that the pathogenicity promoted by this gene is reduced in the absence of adequate expression. Several studies have shown that HlyE pore formation is part of a mechanism for iron acquisition by the bacterial cell or may promote infection by killing immune cells and causing tissue damage (Oscarsson et al., 1999; Lai et al., 2000; Soderblom et al., 2005).

The VF gene *iucD* presented a prevalence of 42%. All isolates of the same clonal type that were sequentially recovered from the same cow were *iucD* positive. This suggests that *iuc* operon, encoding aerobactin, which is involved in iron binding, may be advantageous for the survival and persistence of *E. coli* in the uterus.

Notwithstanding the above considerations, our data showed no relationship between the presence of VF genes and the establishment of the uterine infection. Kaipainen et al. (2002) also did not find an association between the presence of *E. coli* VF genes and the severity of clinical mastitis, but the presence of S and P fimbriae, CNF-1 and CNF-2 were significantly associated with the persistence of mastitis (Lehtolainen et al., 2003).

The *E. coli* in vitro biofilm formation was significantly affected by in vitro conditions. Biofilm formation was greater in a rich medium without salt than in a minimal medium, which contrasts with reports of bacterial adherence and biofilm formation being increased under low nutrition conditions (Yang et al., 2004; Reisner et al., 2006; Naves et al., 2008). Our results may indicate that low osmolarity is more relevant with respect to inducing biofilm formation than a low nutrient level. Prigent-Combaret et al. (2001) observed that the increase in the osmolarity of the medium is responsible for the loss of bacterial adhesion.

Escherichia coli biofilm formation was observed even in strains that did not produce cellulose and did not express curli fimbriae, a finding also reported by Bokranz et al. (2005). This finding shows that these phenotypes are not essential for biofilm formation. A significant association between biofilm formation and curli fimbriae expression and cellulose production was observed only in a minimal nutrient medium. This can explain the persistence of *E. coli* in the environment (manure), which may allow the spread of the same clonal type between animals.

The genes encoding curli fimbriae were present in all isolates, which indicates that differences in phenotype are the result of differences in gene expression, as reported by others (Dyer et al., 2007). Expression of *csgD* is highly regulated (reviewed by Van Houdt and Michiels, 2005). Several environmental factors including nutrient limitation, low osmotic strength, low temperature, microaerophilic conditions, and iron limitation increase the expression of *csgD* (Gerstel and Romling, 2001). Our reported prevalence of expression at 28°C was 83%, which is higher than the 57% reported by Dyer et al., (2007) for *E. coli* strains recovered from bovine mastitis. Our reported prevalences of *E. coli* strains that simultaneously expressed curli fimbriae and produced cellulose (25% at 37°C and 53% at 28°C) are typical values for fecal and commensal isolates (Bokranz et al., 2005; Wang et al., 2006). This supports the idea that *E. coli* isolates recovered from the uterus of puerperal cows are opportunistic, environmental bacteria. In vitro biofilm formation, curli fimbriae expression, and cellulose production were found equally in strains recovered from the uterus of normal puerperium and clinical metritis

cows. Thus, these features are not determinants of uterine infection.

Escherichia coli LIPS is detectable in the uterus, peripheral plasma, and ovarian follicular fluid of cows with postpartum uterine infection (Mateus et al., 2003; Williams et al., 2008), and high concentrations of LPS are related to high concentrations of prostaglandin E₂ (Mateus et al., 2003). Bovine endometrial cells express toll-like receptor 4, which detects LPS and stimulates prostaglandin production (Herath et al., 2006), which ultimately modulates the functional immune response that can lead to the establishment of the infection with other bacteria.

In conclusion, the data presented in this paper indicate that *E. coli* isolates recovered from the uterus of puerperal cows show a wide genetic diversity, do not belong to a known pathogenic group, and have a low potential of virulence and persistence. These observations corroborate the putative role of *E. coli* in the pathogenesis of the puerperal uterine infection: *E. coli* favors the establishment of *Arcanobacterium pyogenes* and gram-negative anaerobes, which in turn favors the establishment and persistence of the infection. It also indicates that other factors, namely the cow's defense mechanisms and the presence of other bacteria, will dictate the establishment and persistence of the uterine infection.

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