



Genetic relationships, biofilm formation, motility and virulence of *Escherichia coli* isolated from bovine mastitis



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Abstract:

Escherichia coli is an environmental bacterium frequently implicated in bovine mammary infections. Although specific virulence factors are unknown, biofilm-forming bacteria are associated with persistent infections, and motile bacteria exhibit increased virulence. An analysis was done of the genetic relationship, biofilm formation capacity and motility of bovine mastitis-associated *E. coli*, as well as the *in vivo* virulence of representative bacterial isolates. Of the 34 isolates, 67.7 % belonged to phylogenetic group A, 17.6 % to group B1 and 14.7 % to group D. Genetic relationship analysis done with (GTG)₅-PCR indicated that the analyzed bacteria are diverse, with only two isolates exhibiting 100 % similarity, and the remaining 32 being grouped into seven main clusters with more than 70 % similarity. Biofilm formation capacity ranged from strong to moderate and weak in 76.5 % of the isolates. The

csgA and *fimA* genes were detected in 52.9 % of the biofilm-forming isolates. Most (70.6 %) of the isolates were motile. *In vivo* infection assays using *Galleria mellonella* showed the biofilm-forming bacteria to be more pathogenic than the non-biofilm-forming ones. The studied bovine mastitis-associated *E. coli* were genetically diverse. Biofilm formation capacity and motility were variable among the isolates, but the biofilm-forming bacteria were more pathogenic than the non-biofilm-forming ones.

Key words: *E. coli*, Bovine mastitis, Diversity, Biofilm, Virulence.

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Introduction

Bovine mastitis is the principal cause worldwide of economic losses in the dairy industry due to decreased milk production, treatment costs, and early disposal of cattle, among other factors⁽¹⁾. *Escherichia coli* is one of the main environmental pathogens associated with bovine mastitis, and its infections can range from moderate to severe^(2,3). The specific virulence factors of bovine mastitis-associated *E. coli* have not been determined to date, but it may form part of a new pathotype known as Mammary Pathogenic *E. coli* (MPEC)⁽⁴⁾. More recent studies at the genomic level have revealed the presence of groups of genes that code for the type VI secretion systems, lipopolysaccharide biosynthesis, biofilm formation and iron uptake systems characteristic of bovine mastitis-associated *E. coli* (BMAEC)⁽⁵⁻⁸⁾. However, other studies suggest that evidence is still insufficient to assign BMAEC to a particular pathotype⁽⁹⁾.

Several phylogenetic analyses of *E. coli* have classified this bacterium into four main groups: A, B1, B2 and D. Non-pathogenic commensal bacteria belong mainly to groups A and B1, although these groups also include important pathogens. Group B2 and a smaller proportion of D contain strains associated with extra-intestinal infections^(9,10). Bovine mastitis-associated *E. coli* (BMAEC) have been assigned mainly to groups A and B1^(11,12,13). In addition, genetic and genomic analyses have revealed that BMAEC are quite diverse; indeed, the *E. coli* associated with both persistent and transient bovine mastitis exhibit both phenotypic and genotypic diversity⁽¹⁴⁾. Genomic analysis of various *E. coli* isolates which cause bovine mastitis and commensals has shown that none can be classified into a particular

phylogenetic group and that in both cases they apparently originated from different lineages⁽⁹⁾.

The ability to colonize and invade the bovine mammary epithelium helps bacteria evade the immune response and infect persistently. The *E. coli* behind persistent mastitis efficiently invades mammary epithelial cells, and, although the mechanisms it uses are not well known, the bacteria exhibiting greater motility also have greater virulence⁽¹⁵⁾. The extracellular polymer matrix of biofilm-forming bacteria makes them more resistant to various chemical substances with antimicrobial activity produced by cells, as well as protecting them from the innate immune system^(16,17).

Bacterial virulence is generally studied using various animal models, although many of these can be expensive and present some ethical problems. A viable and increasingly popular model uses larvae of the greater wax moth *Galleria mellonella* to measure the virulence of Gram positive and negative pathogenic bacteria, as well as fungi^(18,19). For example, *G. mellonella* is a suitable model for study of the pathogenic variants of both intestinal and extraintestinal *E. coli*^(20,21,22).

The present study objective was to analyze the genetic diversity, identify phylogenetic groups, and quantify biofilm formation capacity and motility of *E. coli* isolated from cases of subclinical bovine mastitis, and evaluate the *in vivo* virulence of representative isolates using a *G. mellonella* larvae model.

Material and methods

DNA extract preparation and phylogenetic group identification

The studied *E. coli* were 34 antibiotic-resistant strains isolated from cases of subclinical bovine mastitis in western Michoacán state, Mexico⁽²³⁾. Total DNA extracts were prepared from these isolates⁽²⁴⁾. An established protocol was used to identify the phylogenetic groups of each strain⁽¹⁰⁾. The primers used for fragment amplification were:

ChuA.1 (5'-GACGAACCAACGGTCAGGAT-3');
ChuA.2 (5'-TGCCGCCAGTACCAAAGACA-3');
YjaA.1 (5'-TGAAGTGTCAGGAGACGCTG-3');
YjaA.2 (5'-ATGGAGAATGCGTTCCTCAAC-3');
TspE4C2.1 (5'-GAGTAATGTCTGGGGCATTCA-3');
TspE4C2.2 (5'-CGCGCCAACAAAGTATTACG-3').

The PCR reactions were run using a 25 μ l volume containing: 1X PCR Master mix (Promega, Madison, WI, USA), 1 μ M of each primer and 2 μ l bacterial DNA extract (50 ng). Fragment amplification was done in a C1000 thermocycler (Bio-Rad, Mexico City, Mexico) under the following conditions: initial denaturation at 94 °C for 5 min; 30 cycles as follows, denaturation at 94 °C for 30 s, alignment at 55 °C for 30 s and extension at 72 °C for 30 s; and final extension at 72 °C for 5 min. The amplified products were separated on 1.5% agarose gels and viewed with a Universal Hood II Gel Doc (Bio-Rad).

Biofilm production

Biofilm production was grown according to an established protocol⁽²⁵⁾ with some modifications. Bacteria were seeded in Petri dishes with trypticase soy agar (TSA, BD, Mexico City, Mexico). These cultures were used to inoculate tubes with 2 ml trypticase soy broth (TSB) containing 0.25% glucose, which were incubated overnight at 37 °C under constant agitation. In each tube the cultures were diluted to the 0.5 MacFarland standard with TSB containing 0.25% glucose. From these bacterial dilutions 200 μ l were deposited in triplicate in 96-well ELISA plates and incubated for 24 h at 37 °C without stirring. Non-inoculated culture medium was used as a negative control, and the biofilm-forming bacterium *Pseudomonas aeruginosa* ATCC 27853 was used as a positive control. After incubation the culture medium was discarded, the wells washed three times with sterile saline, and the plate allowed to dry at 60 °C for 1 h to fix the cells. One percent (1%) violet crystal (200 μ l) was added to the wells and the plate left at room temperature for 20 min. It was washed with running water until no color evolution was observed and allowed to dry at room temperature. The violet crystal was solubilized by adding 200 μ l 96% ethanol to each well and stirring, and transferring the supernatant to a microcentrifuge tube. This process was repeated, tube content completed to 1 ml and optical density (OD) of the suspension read at 570 nm with a SmartSpec Plus spectrophotometer (Bio-Rad). This test was repeated four times in triplicate and average OD calculated from the results. The optical density cutoff (OD_c) was calculated using the average OD of the negative control and increasing the value by three times its standard deviation, producing a value of 0.22. The biofilm-forming bacteria were classified into four groups: strong= OD > 4xOD_c; moderate= 2xOD_c < OD ≤ 4xOD_c; weak= OD_c < OD ≤ 2xOD_c; and negative= OD ≤ OD_c⁽²⁵⁾.

Detection of genes associated to biofilm formation

The presence of two genes associated with biofilm formation, *fimA* (main subunit of type I fimbria) and *csgA* (main subunit of curli fimbria), was analyzed by PCR. A 119 bp *fimA* fragment was amplified with *fimA*-F (5'-CTCTGGCAATCGTTGTTCTGTTCG-3') and *fimA*-R (5'-GCAAGCGGCGTTAACAACCTCC-3'), and a 178 bp *csgA* fragment was amplified with *csgA*-F (5'-GATCTGACCCAACGTGGCTTCG-3') and *csgA*-R (5'-GATGAGCGGTCGCGTTGTTACC-3')⁽²⁶⁾. The reactions were run in a 25 µl volume containing 1X PCR Master mix (Promega), 1 µM of each primer, and 2 µl total DNA extract. Amplification conditions were: initial denaturation at 95 °C for 5 min; 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s; and a final extension cycle at 72 °C for 10 min. The amplified products were separated on 2% agarose gels and viewed as described above.

Determination of swarming type motility

Bacterial motility was measured following an established protocol⁽¹⁵⁾. Briefly, swarming motility was determined by allowing the cultures to grow overnight in Lysogeny Broth (LB), and adding 5 µl swarming agar (LB + 0.5% agar) containing 0.5% glucose. After incubating the cultures for 12 h at 37 °C, bacteria displacement diameter was measured. Three measurements were taken and averaged.

Rep-PCR of bovine mastitis-causing *E. coli*

Genetic diversity was analyzed by purifying genomic DNA according to standard protocols⁽²⁷⁾. The purified DNA was amplified in 25 µl reaction mixture containing 12.5 µl 2X Master mix (Promega), 3 mM MgCl₂, 5% DMSO, 0.16 µg/µl bovine serum albumin, 100 ng DNA and 2 µM of the primer (GTG)₅ (5'-GTGGTGGTGGTGGTG-3'). Amplification was done in a C-1000 thermocycler (Bio-Rad) under the following sequence: initial denaturation 95 °C for 2 min; 30 cycles as follows, denaturation (94 °C/3 s and 92 °C/30 s), alignment (40 °C/1 min) and extension (65 °C/8 min); and final extension at 65 °C for 8 min⁽²⁸⁾. The amplified products were separated on 1.5% agarose gels and viewed with a Universal Hood II Gel Doc (Bio-Rad).

Band patterns were analyzed with the GelJ ver. 2 software⁽²⁹⁾. Band size was standardized using a 1 kb DNA ladder (Promega) with fragments between 250 and 10,000 bp as a reference standard. The similarity coefficients were generated by the Dice method with a 2 % tolerance between rows, and the dendrogram built using the UPGMA method.

Virulence in *Galleria mellonella*

In vivo virulence was measured for representative bacteria exhibiting the highest biofilm formation rates, as well as non-biofilm-forming isolates. The *G. mellonella* larvae weighed 150 to 200 mg, were free of apparent damage, exhibited no melanization (necrotic spots) (Petmmal, Cuautitlán Izcalli, Mexico), and were stored at 30 °C in darkness until use. The infection assays were done using bacterial cultures grown in LB broth until the mean exponential growth phase (OD 600 nm= 0.5). These were centrifuged at 12,000 rpm/min, washed twice with 1 ml 10 mM MgSO₄, and resuspended in 1 ml 10 mM MgSO₄. Serial dilutions were made of this cell suspension until attaining a bacterial concentration of approximately 1x10⁵ CFU/ml, which was confirmed by CFU count on LB agar.

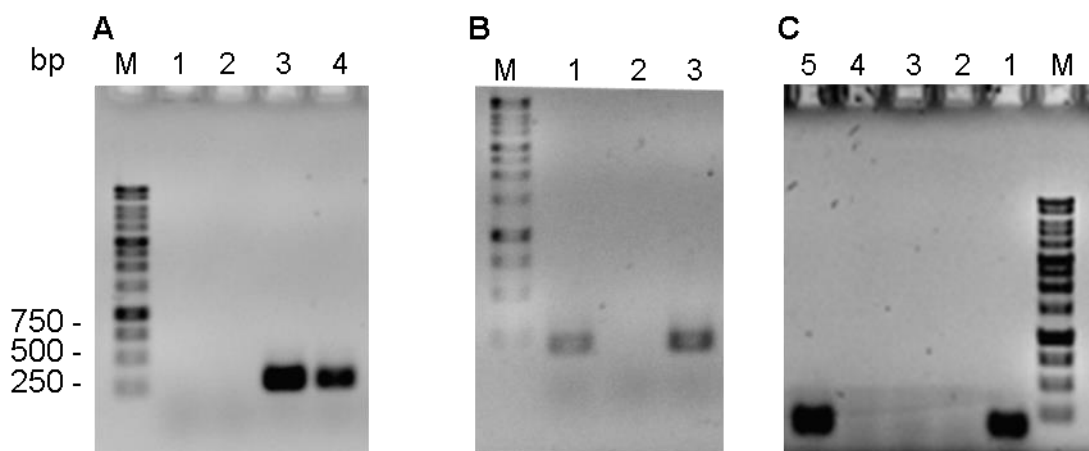
Ten *G. mellonella* larvae were used for each bacterium to be studied. Using an insulin syringe with a 29G gauge needle, 10 µl bacterial suspension (1 x 10⁵ CFU/ml) were injected into the final pro-leg of each larva. The negative control was ten larvae injected with 10 mM of bacteria-free MgSO₄, and an additional ten without inoculation. The inoculated and non-inoculated larvae were placed in sterile Petri dishes and incubated at 30 °C in darkness for 96 h. Survival percentages were measured at 24, 48, 72 and 96 h; larvae unresponsive to touch were recorded as dead⁽²¹⁾. The virulence assay for each bacterium was run at least twice independently.

Results

Phylogenetic groups and genetic relationship of bovine mastitis-associated *E. coli*

Amplification of the *chuA* (279 bp), *yjaA* (211 bp) and TspE4.C2 (152 bp) fragments (Figure 1) showed the 34 isolated BMAEC strains to belong to three phylogenetic groups. Most (67.7 %; 23 isolates) belonged to group A, followed by 17.6 % (6 isolates) in group B1 and the remaining 14.7 % (5 isolates) in group D (Figure 1B).

Figure 1: Agarose gels showing PCR amplification results for A) *chuA* (279 bp), B) *yjaA* (211 bp) and C) TspE.C2 (152 bp)



The genetic relationship analysis by rep-PCR using the (GTG)₅ oligonucleotide yielded 32 different patterns between 7 and 21 bands, with sizes ranging from 250 to 5,000 bp (Figure 2). The dendrogram based on the Dice similarity coefficient and generated by the UPGMA method indicated that the lowest similarity among the isolates was 58 % while the highest was 100 % (in 2 isolates) (Figure 3). Considering a 70 % genetic similarity level (dotted line), seven groups of bacteria were identified; the largest was group VII with 22 isolates (64.7 %), followed by group III with 4 isolates (11.8 %), group VI with 3 isolates (8.8 %), group I with 2 isolates (5.8 %), and groups II, IV and V with a single isolate each (2.9 %).

Figure 2: Agarose gel of products amplified by (GTG)₅-PCR. The base pair size of some bands is indicated by the 1 kb marker (M)

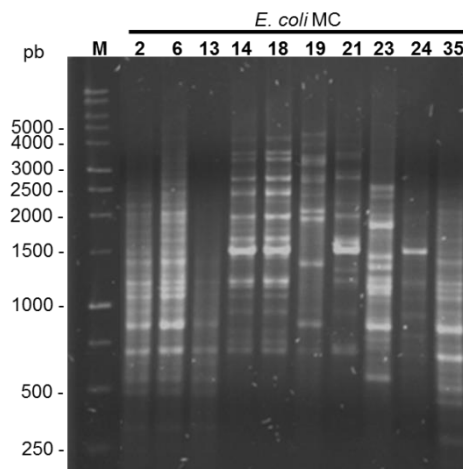
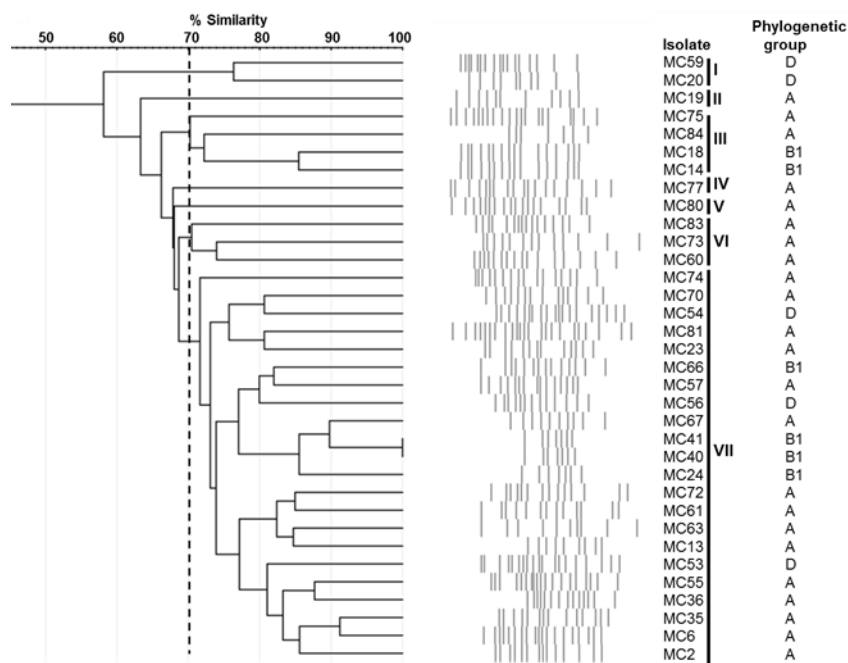


Figure 3: Grouping band patterns for 34 bovine mastitis-associated *E. coli* strains, based on the Dice similarity coefficient and generated by UPGMA



Motility, biofilm formation and associated genes

Ten of the 34 *E. coli* isolates were not mobile (-) and the remaining 24 were mobile. Based on their displacement diameters, twelve of these were minimally mobile (+), six were moderately mobile (++), and six were highly mobile (+++) (Figure 4, Table 1).

Figure 4: Representative examples of “swarming” type motility. A) Not mobile, B) Minimally mobile, C) Moderately mobile, D) Highly mobile

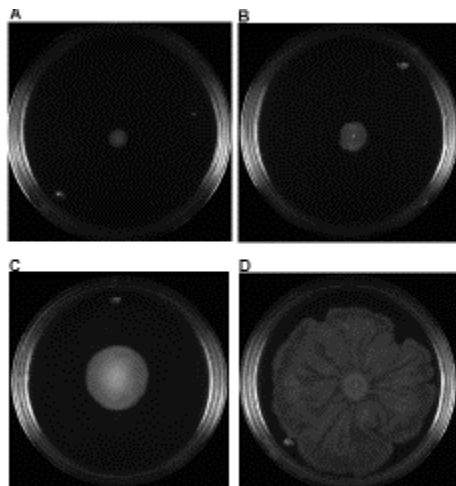


Table 1: Motility, biofilm formation and frequency of biofilm formation-associated genes of the 34 *E. coli* isolates from subclinical bovine mastitis cases

<i>E. coli</i>	Motility ^a	Biofilm ^b	Genotype <i>csgA/fimA</i>
MC75	+++	W	+/+
MC80	+	N	+/+
MC81	+	W	+/+
MC83	+	N	+/+
MC13	-	S	-/-
MC14	-	W	+/+
MC72	+++	M	+/+
MC19	++	S	-/+
MC40	+++	W	+/+
MC41	+	N	+/+
MC59	-	W	-/-
MC73	+	M	-/-
MC66	-	W	+/+
MC2	-	S	-/-
MC6	+	S	-/-
MC35	++	S	-/+
MC36	+	S	-/+
MC84	-	W	-/-
MC24	+	W	+/+
MC56	++	W	+/+
MC67	-	N	+/+
MC74	++	M	-/-
MC77	++	N	-/-
MC55	+	S	-/-
MC60	+	W	-/-
MC54	+++	M	+/+
MC18	+	W	+/+
MC23	-	M	-/+
MC57	-	M	-/-
MC53	+++	N	+/+
MC20	+	N	+/+
MC70	+++	W	+/+
MC61	++	N	+/+
MC63	-	M	-/-

^a(-) not mobile, (+) minimally mobile, (++) moderately mobile, (+++) highly mobile.

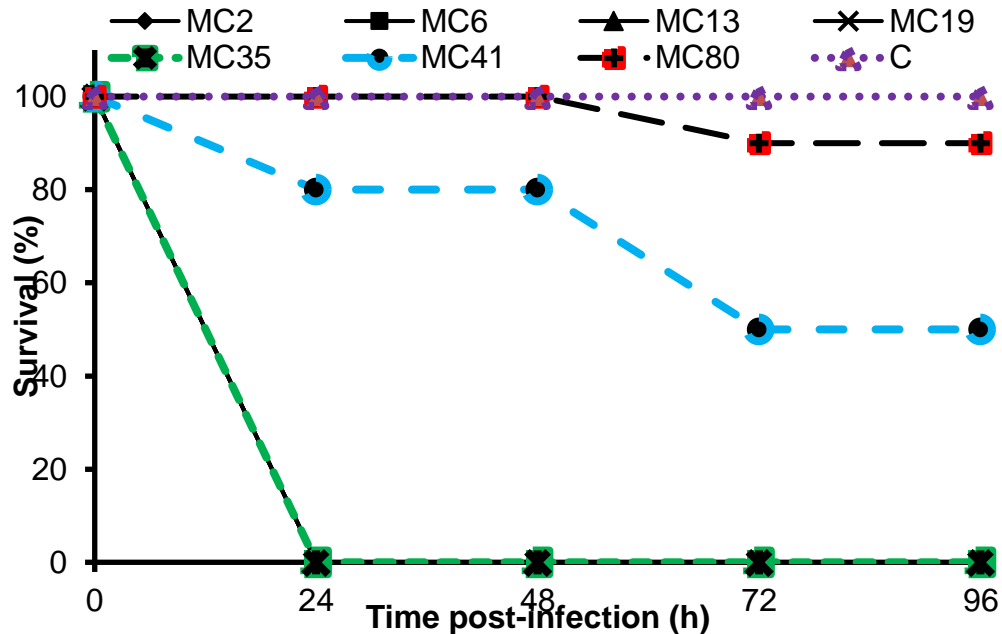
^bN, negative; W, weak; M, moderate; S, strong.

Twenty-six (76.5 %) of the isolates formed biofilm to some degree ($OD > 0.22$) while the remaining eight (23.5 %) did not form biofilm ($OD \leq 0.22$). Of the biofilm-forming isolates seven were classified as having a strong ($OD > 0.88$) and moderate formation capacity ($OD 0.45-0.88$), and twelve as having a weak one ($OD 0.23-0.44$) (Table 1). Twenty-two (64.7 %) of the 34 isolates had either *csgA* or *fimA*, whereas of the 26 biofilm-forming isolates 15 had one or both of these genes. Both *csgA* and *fimA* were present in the seven non-biofilm-forming isolates.

E. coli Virulence in *G. mellonella*

Differences in the pathogenesis of the BMAEC isolates were analyzed by infection trials using *G. mellonella* larvae. Seven representative isolates were tested: five strong biofilm-forming isolates and two non-forming isolates. The biofilm-forming isolates killed 100 % of the larvae 24 h after infection (Figure 5). The two non-biofilm-forming isolates caused from 0 to 20 % mortality at 24 h, and from 10 to 50 % at 96 h. The control resulted in no mortality during the 96-h experiment.

Figure 5: Virulence in *G. mellonella* larvae of a control (C) and seven representative *E. coli* isolates: five biofilm-forming isolates (MC2, 6, 13, 19 and 35), and two non-biofilm forming isolates (MC41 and MC80)



Discussion

The severity of *E. coli*-caused bovine mastitis can range from mild to severe. No specific virulence factors have yet been associated with these disease-causing bacteria^(11,30), and it has even been suggested that infection severity is determined by cattle characteristics rather than by the microorganisms⁽³¹⁾. One line of evidence suggests that mammary gland infections can be caused by any *E. coli* strain⁽¹¹⁾, while another has shown that not all *E. coli* isolates are capable of producing mastitis in animal models⁽⁵⁾.

The *E. coli* strains known to cause bovine mastitis belong mainly to the A and B1 phylogenetic groups, and to a lesser extent to the D group⁽⁷⁾. This coincides with the present results in which 67.7 % of the analyzed isolates belong to phylogenetic group A, 17.6 % to B1 and 14.7 % to D. This overall pattern has been reported previously, with 50 % of the *E. coli* strains associated with both persistent and transient mastitis belonging to the A phylogenetic group, 28.6 % to B1 and 7.1 % to groups B2 and D, respectively⁽¹⁴⁾. In another study 44.88 % of mastitis-associated *E. coli* belonged to phylogenetic group A, 38.58 % to B1 and 16.53 % to D⁽³²⁾. Similar results have also been reported elsewhere^(13,33). In addition, the *E. coli* isolated from bovine mastitis exhibit great diversity in terms of serotype, disease type (transient or persistent) and genotype, even when they belong to the same phylogenetic group^(3,34). The 34 *E. coli* isolates analyzed here also exhibited broad genetic diversity, since only two had identical band patterns and the remaining 32 had similarity ranging from 92 to 58 %. The present results also indicated no clear separation between the different phylogenetic groups, which shows great heterogeneity among the bovine mastitis-causing *E. coli* strains in the study area; this also coincides with previous reports^(3,30,34,35).

The ability to form biofilms in pathogenic bacteria provides them protection against the host immune system and antibiotic resistance, as well as affecting virulence⁽²⁶⁾. The bacteria associated with bovine mastitis manifest a variable pattern of biofilm formation⁽³⁶⁾, although recurrent infections have been associated with biofilm-forming strains⁽¹⁶⁾. In the present results 20.6 % of the isolates formed strong to moderate biofilms, while 35.3 % formed weak ones. Other studies have reported similar results with different BMAEC strains having variable biofilm forming capabilities. In one, 18.5 % of the strains had a strong capacity, while 40.7 % had a moderate to weak capacity⁽³⁶⁾, and in another study 40 % had strong capacity, 12 % had a moderate capacity and 4 % had a weak one⁽³³⁾.

Among the 34 analyzed *E. coli*, the *csgA* and *fimA* genes were detected in 52.9 %, only *fimA* in 11.8 %, and neither gene in the remaining 35.3 %. At least one of the genes was detected in 15 of the 26 biofilm-forming isolates and the seven non-biofilm forming isolates. Both genes have been associated with biofilm formation, as have a variety of other genes and

environmental conditions⁽³⁷⁾. Further research is clearly needed to generate a more detailed characterization of the factors affecting or promoting biofilm formation in the studied *E. coli* isolates. Biofilm formation can also vary in response to strain, culture medium, methodology and quantification method. Only one culture medium and one temperature were tested in the present study, raising the possibility that under different conditions the non-biofilm-forming bacteria that were *csgA*- and *fimA*-positive may be capable of forming biofilms⁽³⁸⁾.

Bacterial motility is another important factor in the spread and production of persistent infections in cattle⁽¹⁵⁾. In the present results 70.6 % of the studied *E. coli* were mobile, suggesting that there were both persistent and transient mastitis bacteria among the studied strains.

The *in vivo* results indicated that the biofilm-forming isolates exhibited greater virulence against *G. mellonella* larvae than the non-biofilm-forming isolates. More study is required to establish the correlation between pathogenesis *in vivo* in *G. mellonella* with the severity of *E. coli*-caused mastitis, but the present results do suggest that this organism is an adequate model for analyzing the pathogenesis of mastitis-causing *E. coli*.

Conclusions and implications

The bovine mastitis-associated *E. coli* analyzed here were genetically and physiologically diverse. The isolates strongly capable of forming biofilms were more pathogenic than the non-forming isolates. The observed genetic and phenotypic diversity indicates that there is no strain, genotype or specific virulence factor associated with mastitis. However, because biofilm-forming bacteria have been associated with recurrent and persistent mammary gland infections, better strategies for controlling infections need to be developed with the aim of reducing the economic losses associated with the consequent reductions in milk production and quality.

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