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*Research article*

# INVESTIGATION OF BIOFILM FORMATION AND PHYLOGENETIC TYPING OF *ESCHERICHIA COLI* STRAINS ISOLATED FROM MILK OF COWS WITH MASTITIS

MILANOV Dubravka*, PRUNIĆ Bojana, VELHNER Maja, TODOROVIĆ Dalibor, POLAČEK Vladimir

Scientific Veterinary Institute „Novi Sad", Novi Sad, Republic of Serbia

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*Escherichia coli* is an opportunistic pathogen affecting bovine mammary gland causing mainly transient infections; however, some recent reports indicated that some strains are able to adhere to and internalize into the epithelial cells, which can result in the persistence of the pathogen in the tissue and development of recurrent mastitis. The mechanism of adaptation of *E. coli* to the mammary gland relies on structures that are distinctive components of its extracellular matrix - *curli* fimbriae (proteinaceous component) and cellulose (polysaccharide). Expression of these components varies among the isolates. In this study, we investigated the capacity of expression of *curli* fimbriae and cellulose (via colony morphotype on Congo Red agar) and ability of biofilm formation (microtiter plate test) in 25 strains of *E. coli* isolated from milk of cows with clinical mastitis. Phylogenetic grouping of the isolates was performed using PCR method based on detection of *chuA*, *yjaA* and TspE4-C2 amplicons. Antimicrobial susceptibility was examined using standard disk diffusion test. Production of both extracellular matrix components was established in 56%, and expression of *curli* fimbriae in 64% *E. coli* isolates. All isolates that produced *curli* fimbriae, demonstrated this ability at a temperature of 37°C, indicating the potential role of these adhesive structures in the pathogenesis of mastitis. The results of phylogenetic typing confirmed that *E. coli* strains isolated from milk of cows with mastitis are typical commensals mainly belonging to phylogenetic groups A and B1. All *curli* and *curli*/cellulose producing isolates formed biofilm under *in vitro* conditions. The biofilm potentially plays an important role in the development of persistent infections as well as recurrent clinical symptoms after antibiotic therapy in spite of quite good *in vitro* antimicrobial susceptibility of the agent.

**Key words:** biofilm, *curli* fimbriae, cellulose, *Escherichia coli,* mastitis

## INTRODUCTION

*Escherichia coli* are genetically highly versatile bacterial species, which can cause a range of intra- and extraintestinal diseases in both humans and animals. Specific gene groups and virulence factors are responsible for the colonization, multiplication and survival

*Corresponding author: e-mail: dubravka@niv.ns.ac.rs

of the organism within the host body. Based on the presence and detection of such factors, several pathotypes of *E. coli* were identified, which are associated with the three general clinical syndromes: enteric/diarrhoeal disease, urinary tract infection and sepsis/meningitis [1]. In *E. coli* bovine mastitis isolates, diverse virulence factors were established both individual and combinations thereof; however, their assignment into the separate new pathotype has not yet been justified [2-5].

*Escherichia coli* belongs to the group of environmental agents of bovine mastitis, which are not naturally adapted to survival within the host and cause only transient infections by being effectively eliminated from the host body [6]. However, since 1979, when persistent *E. coli* infection of the udder was first described, there has been growing evidence on the altered nature of coli mastitis. Individual *E. coli* strains manifest a certain ability to adapt to the bovine mammary environment and are capable to induce persistent intramammary infections (IMI) [3, 6-9]. Thus, recurrent clinical coliform mastitis might be attributed not only to reinfection, but to the persistence of the organism within the mammary gland as well [8]. Examination of the mammary gland bioptates originating from cows naturally infected with persistent *E. coli* strain revealed the presence of bacteria inside the mammary epithelial cells and mammary duct, whilst in vitro investigations demonstrated that persistent *E. coli* invade and survive in the mammary cell culture more effectively than strains causing transient infections [10]. Intracellular positioning of the pathogen protects the organism from the activity of host's immune system effectors and antibiotics.

Adhesion and internalization of *E. coli* into host epithelial cells is mediated by cell surface structures such as flagella and pili. Type 1 fimbriae are the most common adhesive organelles of *E. coli*, which mediate the adhesion of the organism to the host's mannose-containing glycoproteins [5,10]. Another type of cell surface filaments, the curli fimbriae, was first described in the late 1980s in *Escherichia coli* strains that caused bovine mastitis, and subsequently also in other *Enterobacteriaceae* (*Salmonella*, *Shigella*, *Citrobacter*, *Enterobacter*) organisms [11,12]. Genes mediating the synthesis of curli fimbriae are ubiquitous in *E. coli*, yet their synthesis-capacity varies depending on a range of environmental factors. In that respect, not all *E. coli* strains are able to produce curli fimbriae [5,11,13]. Curli fimbriae play an important role in the initial stages of the infection process by mediating adhesion and invasion of host cells [5,11]. After internalization into the host's epithelial cells, uropathogenic *E. coli* (UPEC) form intracellular bacterial communities (IBCs) manifesting biofilm behaviour, which might be of importance for the pathogenesis of persistent *E. coli* bovine mastitis [14-16]. Besides the specific pattern of tissue adhesion, the production of exopolysaccharides as the principal component of *E.coli* matrix is of the paramount importance for the biofilm formation. *E. coli* produces several exopolysaccharides, such as colanic acid (or M antigen), cellulose, β-1,6-N-acetyl-D-glucosamine (PGA) [12,13]. The in vitro production of curli fimbriae and cellulose is usually tested using the colony morphotypes on Congo Red agar. Both components are capable of interacting with diazo dye Congo Red, which results in the production of rdar (red, dry and rough)

colonies of typical dark purple colour, with rough and dry surface and undulate margins [17]. Rdar morphotype was first described in *Salmonella* Typhimurium [18] and is used as a synonym for multicellular bacterial communities expressing curli fimbriae and cellulose and characterised by an extensive biofilm formation on abiotic surfaces [17].

To the best of our knowledge, the expression of the two principal components of the extracellular matrix, that is, curli fimbriae (proteinaceous component) and cellulose (polysaccharide component) as well as the biofilm formation in vitro, have not yet been investigated in bovine mastitis isolates of *Escherichia coli* in our country. Based on a justified hypothesis that these characteristics might significantly affect the outcome of mastitis, i.e. healing of bacterial infection or persistence of the strain in the udder, the research on biofilm formation ability in *E. coli* bovine mastitis isolates was conducted. In addition the expression of curli fimbriae and cellulose was identified visualizing the colony morphotype on Congo Red agar.

## MATERIALS AND METHODS

### *Bacterial isolates, identification and storage*

The investigation encompassed 25 strains of *E. coli* isolated from milk of cows with clinical mastitis on the territory of South Bačka District during 2013. Milk samples originated from cows from different small holdings (n=7) and two large dairy farms of Holstein-Friesian breed (n=18). Milk samples were collected applying the aseptic technique and processed immediately after submission to the laboratory. Columbia agar supplemented with 5% sheep blood and MacConkey agar (CM0007,Oxoid, Basingstoke, UK) were inoculated with 50 mL of milk and incubated for 24-48h at 37°C under aerobic conditions. For further investigation, only pure cultures of *E. coli* strains isolated from apparently changed milk samples (yellow watery secretion with flakes and clots) were used. Identification of the isolates was performed using BBL Crystal Enteric/Nonfermenter test (Becton Dickinson, Detroit, MI, USA). Until testing, the strains were stored in Tryptone soya broth (TSB) (CM0129, Oxoid, Basingstoke, UK) with 15% glycerol at -80°C. Overnight cultures cultivated on MacConkey agar at 37°C were used for the examination.

### *Phylogenetic typing of E. coli*

Of the cultures grown on MacConkey agar, 2-3 single colonies were harvested and inoculated into Tryptone soya broth (CM0129, Oxoid, Basingstoke, UK) to obtain the overnight cultures. The DNA was isolated by boiling procedure. PCR was done with 3 sets of primers according to Clermont *et al.* [19], for the following genes: *chuA*, *yjaA* and anonymous DNA fragment TspE4-C2. The sequence of the target genes are as follows: ChuA.1 (5`-GAC GAA CCA ACG GTC AGG AT-3`) and ChuA.2 (5`-TGC CGC CAG TAC CAA AGA CA-3`), YjaA.1 (5`-TGA AGT GTC AGG AGA CGC

TG-3`) and YjaA.2 (5`-ATG GAG AAT GCG TTC CTC AAC-3`), TspE4C2.1 (5`-GAG TAA TGT CGG GGC ATT CA-3`) and TspE4C2.2. (5`-CGC GCC AAC AAA GTA TTA CG-3`). Cycling conditions were: denaturation 15 min at 95°, 30 cycles of 30s at 94°C, 30s at 55°C and 30s at 72°C, final extension step of 7 min at 72°C. Master mix in volume of 25 μl was prepared according to manufacture instruction (Qiagen, Hilden, Germany). This method enabled identification of four phylogenetic groups - A, B1, B2 and D. The results were interpreted as follows: phylogenetic group A is assigned if none or *yjaA* PCR products were obtained, group B1 is *TspE4-C2* positive, group D is positive to *chuA* or *chuA* and *TspE4-C2* genes, while group B2 was either positive to all 3 genes or to *chuA* and *yjaA*.

### Congo Red agar test

Congo Red agar (CRA) was prepared from Luria Bertani broth (LB) without salt: Bacto Yeast Extract (5g/L), Bacto Tryptone (10 g/L) (Becton, Dickinson and Company), supplemented Congo Red (40 mg/L) and Comassie brilliant blue (20 mg/L). Colonial morphology was checked after 72h of incubation at 20°C and 37°C. The results were interpreted on the basis of characteristic morphotype characteristics described in *S.* Typhimurium: rdar (red, dry, and rough; expresses curli and cellulose), pdar (pink, dry, and rough; expresses cellulose), bdar (brown, dry, and rough; expresses curli), and saw (smooth and white; neither curli nor cellulose) [17,20], saw/mucous (capsule production) and less-pronounced phenotype described by Bokranz *et al.* [21]: ras (violet and smooth; curli only), bas (brown and smooth; curli only) or pas (pink and smooth; cellulose only).

### Microplate biofilm assay

#### *Preparation of the inoculum*

Individual colonies (up to 1mm in diameter) of cultures grown on MacConkey agar were harvested, inoculated into 5mL buffered peptone water (CM1049, Oxoid, Basingstoke, UK) and then incubated for 18h at 30°C. The inoculums for the microplate test were prepared by diluting this overnight suspension in fresh TSB and LB without salt at the ratio 1:40. The density of the prepared inoculums was determined applying standard technique of cell-count in tenfold dilution, being 2-8 x $10^7$ CFU/mL.

#### *Microplate biofilm assay procedure*

An aliquot of 200 μL of LB and TSB suspensions prepared from each strain were inoculated into the wells (4 wells for each strain) of sterile flat-bottom microtiter plates (Nunc, Rosklide, Denmark) and incubated for 48h at 20°C and 37°C. After incubation, the plates were washed three times with sterile saline (250 μL per each well) and allowed to dry at room temperature in an inverted position. Fixation with methanol (250 μL per well) was performed for 20 minutes at room temperature with subsequent

staining with 0,3% crystal violet (No. 42555, Sigma-Aldrich), 250 μL per well during 15 minutes at room temperature. The plates were washed out under running water until there were no visible traces of colour. At this stage, pellicle formation was inspected visually. The bound stain was dissolved with 95% ethanol (250 μL per well) during 15 minutes at refrigerator temperature. The test was performed twice. The optical density (OD) was measured spectrophotometrically (Labsystems Multiscan® MCC/340) using 595nm filter. Cut-off OD (ODc) is defined as three standard deviations above the mean OD of the negative control. Strains were classified as follows: non-biofilm producers (OD ≤ ODc); weak biofilm producers (ODc < OD ≤ 2 x ODc); moderate biofilm producers (2 x ODc < OD ≤ 4 x ODc) and strong biofilm producers (OD > 4 x ODc) [22].

### *Antibiotic susceptibility testing*

Susceptibility of bovine mastitis isolates of *E. coli* to antibiotics was tested using standard disc diffusion test in Mueller Hinton agar (CM0337, Oxoid, UK) in line with the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (documents M100-S22 and M07-A9, 2012). The following antibiotics were used: amoxicillin/clavulanic acid (AMC, 20/10 μL), ampicillin (AMP,10 μL); cefpodoxime (CPD,10 μL); ceftazidime (CAZ, 30 μL); cefotaxime (CTX, 30 μg); ciprofloxacin (CIP, 5 μL); chloramphenicol (CHL, 30 μL); gentamycin (GM, 10 μL); nalidixic acid (NA, 30 μL); streptomycin (S, 10 μL); sulphonamide (SSS, 300 μL); tetracycline (TET, 30 μL); trimethoprim (TMP, 5 μL); trimethoprim +sulfamethoxazole (SXT, 1.25/23.75 μL) (susceptibility discs, Bio-Rad, France).

## RESULTS

### *Phylogenetic typing*

Twenty-five bovine mastitis isolates of *E. coli* were assigned into the following phylogenetic groups: A (n=16), B1 (n=8) and D (n=1) (Table 1). Commensal non-pathogenic *E. coli* was assigned to groups A and B1, while extraintestinal *E coli* belong exclusively to group B2 and partially to group D. Group D is constituted from extraintestinal-pathogenic and commensal none-pathogenic *E. coli* strains.

**Table 1.** Phylogenetic typing of 25 isolates of *Escherichia coli* from mastitis milk samples obtained by PCR (primers: ChuA, 279bp, YjaA, 211bp, TspE, 152bp)

| Isolates | Primer | | | Phylogenetic group |
|---|---|---|---|---|
| No. | ChuA.1 and ChuA2. | YjaA.1 and YjaA.2 | TspE4C2.1 and TspE4C2.2 | |
| 1 | - | - | - | A |
| 2 | - | - | + | B1 |
| 3 | - | - | + | B1 |
| 4 | - | - | - | A |
| 5 | - | - | + | B1 |
| 6 | - | - | + | B1 |

cont. Table 1.

| | | | | |
|---|---|---|---|---|
| 7 | - | - | - | A |
| 8 | - | - | + | B1 |
| 9 | - | + | - | A |
| 10 | - | + | - | A |
| 11 | - | - | - | A |
| 12 | - | + | - | A |
| 13 | - | - | - | A |
| 14 | - | + | - | A |
| 15 | - | + | - | A |
| 16 | - | - | + | B1 |
| 17 | - | + | - | A |
| 18 | - | - | - | A |
| 19 | - | - | + | B1 |
| 20 | - | + | - | A |
| 21 | - | + | - | A |
| 22 | - | + | - | A |
| 23 | - | - | - | A |
| 24 | - | - | + | B1 |
| 25 | + | - | - | D |

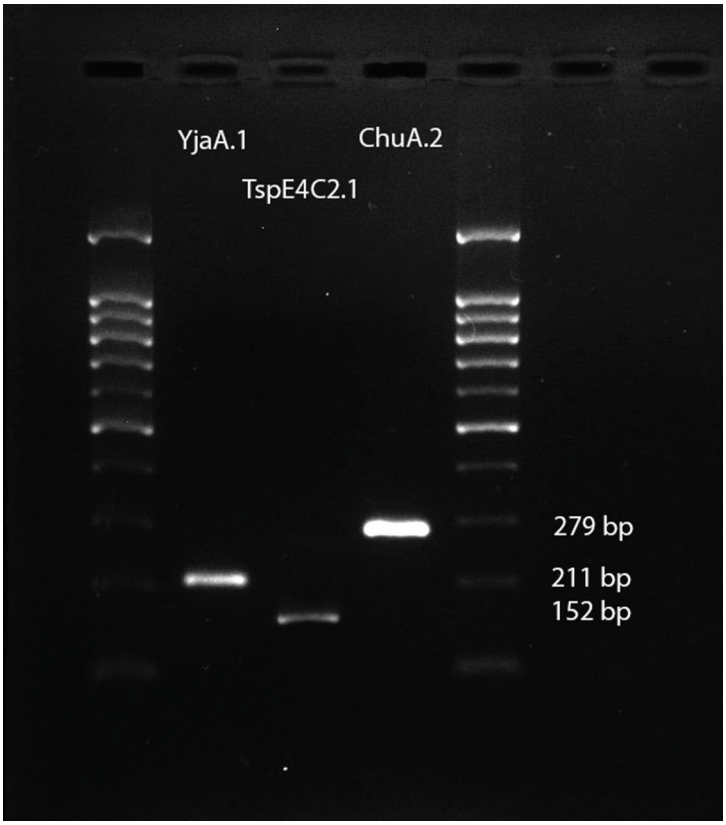


**Figure 1.** The amplicons obtained during phylogenetic typing

### Congo Red agar test

After 72-h incubation period, the rdar morphotype was identified in 11 (44%) isolates at both temperature values (20 and 37°C). Two isolates produced rdar morphotype

**Table 2.** Morphotypes on Congo Red plates

| Isolates No. | CRA morphotypes | | Isolates No. | CRA morphotypes | |
|---|---|---|---|---|---|
| | 20°C | 37°C | | 20°C | 37°C |
| 1 | rdar | rdar | 14 | rdar | bdar |
| 2 | rdar | rdar | 15 | saw | bas |
| 3 | rdar | rdar | 16 | rdar | rdar |
| 4 | rdar | rdar | 17 | saw | bas |
| 5 | rdar | rdar | 18 | rdar | rdar |
| 6 | ras | rdar | 19 | rdar | rdar |
| 7 | saw | saw | 20 | mucoid | mucoid |
| 8 | saw | saw | 21 | saw | saw |
| 9 | saw | saw | 22 | mucoid | mucoid |
| 10 | rdar | ras | 23 | rdar | rdar |
| 11 | rdar | rdar | 24 | saw | saw |
| 12 | saw | saw | 25 | saw | saw |
| 13 | rdar | rdar | | | |

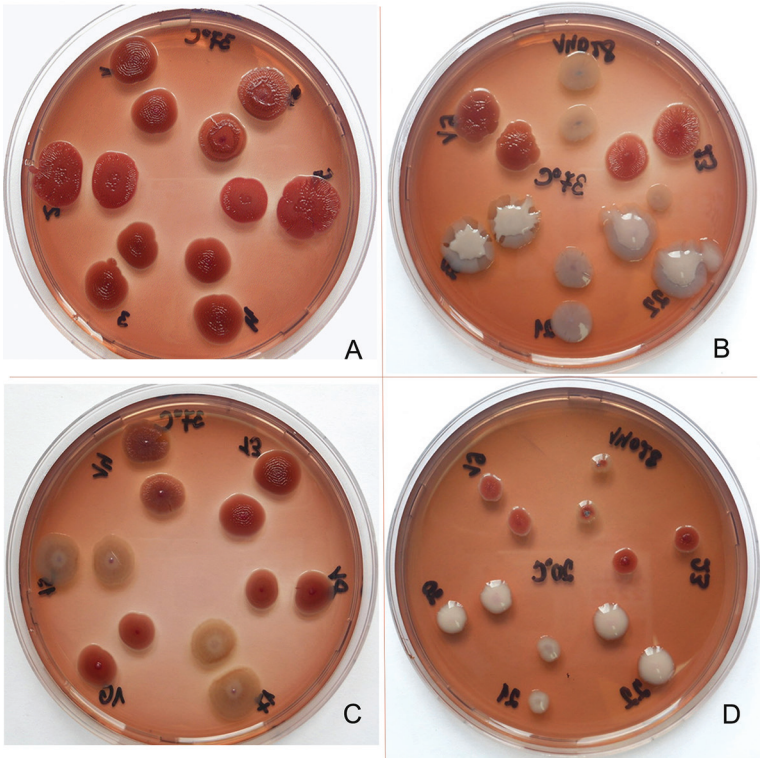


**Figure 2.** Morphotypes of *E. coli* isolates on Congo Red agar, 72-h incubation at 37°C (A-C) and 25°C (D). **(A)** rdar morphotypes; **(B)**: rdar, saw and saw/mucous morphotypes; **(C)** bdar,rdar, and ras morphotypes; **(D)**: rdar, saw and saw/mucous morphotypes)

only at 20°C, and one isolate only at 37°C. The expression of curli fimbriae, which correlated with rdar, ras, bdar and bas morphotypes, was established in 16 (64%) isolates, whereby all isolated produced curli fimbriae at 37°C. Neither pdar (pink and rough) nor pas (pink and smooth) morphotypes that are characteristic for strains that produce cellulose but not curli fimbriae, were identified. At both incubation temperatures, 9 (36%) *E. coli* isolates produced neither curli fimbriae nor cellulose (7 saw-morphotypes and 2 saw/mucous). The colonial morphotype on CR agar is presented in Table 2, and characteristic colonial morphotypes on CRA in Figure 2.

### *Microplate biofilm assay*

The established values for optical density (OD) using crystal violet microplate test are presented in Table 3. After incubation in LB at 20°C, 13 (52%) bovine mastitis *E. coli* isolates were categorized as strong biofilm producers, 3 (12%) isolates as weak biofilm producers, whereas 9 isolates (36%) did not produce biofilm. Following incubation in LB at 37°C, 10 (40%) isolates were assessed as strong, 3 (12%) as moderate, 1 (4%) as weak biofilm producers, whereas 11 (44%) isolates did not produce biofilm. After incubation in TSB at 20°C, 11 (44%) isolates were identified as strong, 5 (20%) as moderate, 1 (4%) as weak biofilm producers, while in 8 (32%) isolates the production of biofilm did not occur. After incubation in TSB at 37°C, 19 (76%) isolates did not produce biofilm, and 6 (24%) were classified as weak biofilm producers (Table 3).

**Table 3.** Results of the microplate biofilm assay

| | Luria Bertani | | | | Triptone soy broth | | | |
|---|---|---|---|---|---|---|---|---|
| | **20 °C** | **%** | **37 °C** | **%** | **20 °C** | **%** | **37 °C** | **%** |
| Strong biofilm producer | 13 | (52) | 10 | (40) | 11 | (44) | 0 | (0) |
| Moderate biofilm producer | 0 | (0) | 3 | (12) | 5 | (20) | 0 | (0) |
| Weak biofilm producer | 3 | (12) | 1 | (4) | 1 | (4) | 6 | (24) |
| Non biofilm producer | 9 | (36) | 11 | (44) | 8 | (32) | 19 | (76) |

– non biofilm producer: OD ≤ 0.318;
– moderate biofilm producer: 0.636 < OD ≤ 1.272
– weak biofilm producer: 0.318 < OD ≤ 0.636
– strong biofilm producer: OD > 1.272

### *Antibiotic susceptibility testing*

Twenty (80%) *E. coli* isolates from bovine mastitis manifested susceptibility towards all antibiotics used in this investigation (Table 4). One isolate demonstrated resistance to only S and TET, whereas multiple antimicrobial resistance was established in four isolates. Resistotype of the isolates is displayed in Table 5.

**Table 4.** Zone Diameter Interpretive Standards for *Eneterobacteriaceae* and the results of the antibiotic susceptibility testing of *E. coli* bovine mastitis isolates

| Antimicrobial Agent | Disk Content | Zone Diameter Nearest Whole mm | | | No of isolates *E. coli* | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Resistant | | Intermediate | | Sensitive | |
| | | R | I | S | No | % | No | % | No | % |
| Ampicillin | 10 µg | ≤13 | 14-16 | ≥17 | 4 | 16.0 | 0 | 0 | 21 | 84.0 |
| Amoxicillin-clavulanic acid | 20/10 µg | ≤13 | 14-17 | ≥18 | 0 | 0 | 0 | 0 | 25 | 100 |
| Cefpodoxime | 10 µg | ≤17 | 18-20 | ≥21 | 0 | 0 | 0 | 0 | 25 | 100 |
| Ceftazidime | 30 µg | ≤14 | 15-17 | ≥18 | 0 | 0 | 0 | 0 | 25 | 100 |
| Cefotaxime | 30 µg | ≤14 | 15-22 | ≥23 | 0 | 0 | 0 | 0 | 25 | 100 |
| Ciprofloxacin | 5 µg | ≤15 | 16-20 | ≥21 | 3 | 12.0 | 0 | 0 | 22 | 88.0 |
| Chloramphenicol | 30 µg | ≤12 | 13-17 | ≥18 | 0 | 0 | 0 | 0 | 25 | 100 |
| Gentamycin | 10 µg | ≤12 | 13-14 | ≥15 | 1 | 4.0 | 0 | 0 | 24 | 96.0 |
| Nalidixic acid | 30 µg | ≤13 | 14-18 | ≥19 | 4 | 16.0 | 0 | 0 | 21 | 84.0 |
| Streptomycin | 10 µg | ≤11 | 12-14 | ≥15 | 5 | 20.0 | 0 | 0 | 20 | 80.0 |
| Sulphonamides | 300 µg | ≤12 | 13-16 | ≥17 | 4 | 16.0 | 0 | 0 | 21 | 84.0 |
| Tetracycline | 30 µg | ≤11 | 12-14 | ≥15 | 4 | 16.0 | 1 | 4.0 | 20 | 80.0 |
| Trimethoprim | 5 µg | ≤10 | 11-15 | ≥16 | 4 | 16.0 | 0 | 0 | 21 | 84.0 |
| Trimeth.-sulfamethoxazole | 1.25/23.75 µg | ≤10 | 11-15 | ≥16 | 4 | 16.0 | 0 | 0 | 21 | 84.0 |

**Table 5.** Resistotype of E. coli bovine mastitis isolates

| Isolates No. | Resistotype: |
|---|---|
| 18 | S, TET |
| 20 | AMP, NA, S, SSS, TET, TMP, SXT |
| 21 | AMP, CIP, GM, NA, S, SSS, TET, TMP, SXT |
| 22 | AMP, CIP, NA, S, SSS, TMP, SXT |
| 25 | AMP, CIP, NA, S, SSS, TET, TMP, SXT |

## DISCUSSION

*Escherichia coli* manifest an excellent ability to adapt to variable environmental niches including the bovine mammary gland. Although coli-mastitis has been considered a transient infection implying both clinical healing and elimination of the agent from the tissue, some strains are capable of causing persistent intramammary infection (IMI). The prevalence of persistent IMI ranges from 5 to 24% of all coli mastitis cases [10]. The pathogenesis of persistent IMI has not yet been fully elucidated; however, the role of serum resistance, survival of bacteria in neutrophils, capacity of internalization into mammary epithelial cells and biofilm behaviour have been the topic of recent research [3,6,10,15]. Persistent infection involves the adhesion, invasion and intracellular survival

[10]. Curli fimbriae are structures mediating internalization into mammary epithelial cells. Their synthesis in *Enterobacteriaceae* is a temperature-dependent phenomenon. In *Salmonella* species, the synthesis of curli fimbriae is usually observed at temperatures below 30°C, whereas enteropathogenic, enterotoxigenic and uropathogenic *E. coli* (EPEC, ETEC and UPEC) express curli fimbriae only at ambient temperature. Enterohaemorrhagic and enteroinvasive *E. coli* (EHEC, EIEC) commonly do not express curli fimbriae in vitro [21]. In bovine mastitis cases investigated in this study, the expression of curli fimbriae was established in 64% of isolates at both incubation temperatures (20°C and 37°C). The ability of *E. coli* isolates from bovine mastitis to express curli fimbriae at 37°C indicate that these adhesins may be involved in the pathogenesis of bovine mastitis [23]. The expression of curli fimbriae at 37°C was established in the majority of human sepsis and clinical isolates of *E. coli* [11,13] as well as in commensal *E. coli* strains isolated from the faeces of healthy humans [21]. The characteristic rdar-morphotype characterized by both expression of curli fimbriae and cellulose production was identified in 14 isolates (56%), whereby 11 (44%) isolates produced rdar-morphotype at both incubation temperatures. Zogaj *et al.* [18] reported that some *E. coli* isolates produce cellulose as a component of the extracellular matrix; however, some other components other than cellulose may also play an important role in the production of rdar phenotype, as was established in *E. coli* O157:H7 [24].

Neither of *E. coli* isolates produced merely cellulose, i.e., pdar and pas morphotypes were not indentified on CR agar. Cellulose synthesis is typical for species such as *Salmonella enterica* subsp. *enterica* serovars Typhimurium and Enteritidis, but some commensal and pathogenic strains of *E. coli*, *Citrobacter* spp. and *Enterobacter* spp. are capable of producing cellulose too [12]. All curli and curli/cellulose producing *E. coli* isolates (64%) formed biofilm in vitro. Fernandes *et al.* [15] also reported in vitro biofilm formation in all *E. coli* isolates from bovine mastitis (n=27), albeit to different extents. Lower incubation temperature (20°C) positively affected biofilm formation in 64% isolates (total of strong and weak biofilm producers) when cultivated in LB, and in 68% isolates cultivated in TSB (total of strong, moderate and weak biofilm producers). Higher incubation temperature of 37°C did not significantly influence the ability of biofilm production compared to cultivation of strains in low-nutrient media – LB, 56% of biofilm producers (total of strong, moderate and weak biofilm producers) (Table 3). However, the cultivation in rich nutritive medium (TSB) at 37°C manifested pronounced inhibitory effect on biofilm formation in all strains (Table 3). Stepanović *et al.* established that 1/20 TSB (nutrient limited medium) was the most effective medium for biofilm formation at 35°C in *Salmonella* spp. [25]. Also in other Gram-negative bacteria, such as *Escherichia coli*, the formation of a biofilm occurs more rapidly when growing under aerobic condition in low-nutrient media, which is due to maximal expression of agfD promoter in nutrient limited medium [25]. *E. coli* isolates did not manifest any visible adherence on the bottom of microplate wells (inspected visually after washing off crystal violet stain), but all moderate and strong biofilm producers produced clearly visible pellicles at the air-liquid interface. Both

components of the extracellular matrix play a role in the adhesion to inert surfaces and formation of the biofilm in vitro. Curli fimbriae are essential during the initial stage of biofilm formation, likely in the attachment phase [5,11,24] as well as in cell-cell interactions (cell aggregations) [5,11,12], whereas biofilm formation at the air-water interface is associated with cellulose expression [12].

Thus, it is not surprising that all curli and curli/cellulose producing strains formed biofilm. Rdar phenotype of *E. coli* was associated with biofilm formation on glass and polystyrene surface as well as the pellicle formation at the air-liquid interface [24]. Contrary to rdar, bdar, bas and ras morphotypes, the biofilm formation did not occur in isolates producing saw and mucous colonies on CRA, although the biofilm behaviour in *E. coli* isolates is observed even when cellulose and curli fimbriae were not expressed [21]. The analysis of capsular polysaccharides of *E. coli* (group II capsular polysaccharides) revealed that they antagonize biofilm formation by reducing initial bacterial adhesion to the surface and inhibiting the cell-cell interaction, i.e. the processes essential for biofilm maturation [12].

The majority of *E. coli* isolates from bovine mastitis in this study were identified as members of phylogenetic group A (64%), which encompasses most commensal (non-pathogenic) strains of *E. coli*. Thirty-two percent of the isolates were categorized into phylogenetic group B1, whereas only one isolate (from a private farm) was classified into group D. The results of this phylogenetic typing correspond well with the results reported by other researchers, suggesting that majority of *E. coli* strains causing transient and recurrent mastitis belong to phylogenetic groups A and B1 and only a small percentage is classified into groups B2 and D defining them as typical commensals [10,15,26]. In a standard disc diffusion test, 20 (80%) *E. coli* isolates manifested susceptibility to all examined antibiotics (Table 4). *E. coli* isolates from bovine mastitis do not show a particular pattern of resistance to antibiotics [3,4]. Only one biofilm-forming isolate with rdar colonial morphotype (No.18) demonstrated resistance to streptomycin and tetracycline, which are widely used antibiotics in the therapy of mastitis. However, in spite of good in vitro antimicrobial susceptibility of all biofilm-forming isolates of *E. coli* it is well established that bacteria in a biofilm demonstrate even 1000 times higher resistance to antibiotics. The recurrence of clinical symptoms after antibiotic therapy in spite of good in vitro susceptibility to antibiotics might be attributed to the persistence of the biofilm of *E. coli* in bovine mammary environment [16,27].

Some recent researches demonstrated that sub-inhibitory concentrations of antibiotics such as aminoglycosides [28] and fluoroquinolones (enrofloxacin) [16] induce *E. coli* biofilm formation. Administration of antibiotics in the therapy of coli mastitis is anyway controversial and justified only in the prevention of bacteraemia (sepsis). Multiple resistance to antibiotics was established in isolates that neither formed biofilm nor expressed curli fimbriae and cellulose (saw and saw/mucous morphotypes) (Table 5). All multiple resistant isolates originated from cows from private farms.

Mastitis is still the leading health and economic problem in dairy herds worldwide, despite continuous efforts in finding new solutions in the field of prevention, treatment and control. In our country, mastitis is a highly attractive field of scientific interest, from several aspects such as virulence factors and molecular mechanisms of antimicrobial resistance [29], immune mechanisms in the mammary glands of cows - lactoferrin gene genotypes [30], and analysis of prophylactic efficacy of vaccines [31]. Some recent results significantly modified the approach to understanding the pathogenesis of certain infections. Thus, the formation of biofilms in some isolates of *Escherichia coli*, provides a different insight into the dynamic processes of bacterial invasion, adherence and persistence, and draws some parallels in relation to infections caused by *S. aureus.*

## CONCLUSION

The expression of curli fimbriae and cellulose, which are associated with the organism's virulence, as well as the ability of biofilm production that was observed in the majority of *E. coli* isolates from bovine mastitis, might play an important role in spreading of biofilm-forming strains in the environment, milking systems, as well as in the interaction with mammary epithelial cells and occurrence of persistent coli mastitis. Particular subset of *E. coli* substantially adapted to the mammary environment (yet unidentified) might be expected. Moreover, potential presence of mastitis *E. coli* pathotype should be taken into consideration [4,8]. Multicellular behaviour pattern (biofilm communities) of *E. coli* isolates from bovine mastitis might be of importance in strain persistence in the bovine mammary environment and recurrent mastitis thus emphasizing the need for further investigation and novel approaches in understanding the pathogenesis of coli mastitis in dairy cattle.

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## ISPITIVANJE FORMIRANJA BIOFILMA I FILOGENETSKA TIPIZACIJA SOJEVA *ESCHERICHIA COLI* IZOLOVANIH IZ MLEKA KRAVA SA MASTITISOM

MILANOV Dubravka, PRUNIĆ Bojana, VELHNER Maja, TODOROVIĆ Dalibor, POLAČEK Vladimir

*Escherichia coli* je oportunistički patogen za mlečnu žlezdu krava koji uglavnom izaziva prolazne infekcije, ali je od nedavno poznato da pojedini sojevi imaju sposobnost adhrencije i internalizacije u epitelne ćelije, što može rezultirati perzistencijom patogena u tkivu i pojavom rekurentnih mastitisa. Mehanizam adaptacije *E. coli* na mlečnu žlezdu uključuje strukture koje su glavne komponente ekstracelularnog matriksa i čija je ekspresija varijabilna osobina među izolatima: *curli* fibrije (proteinska komponenta) i celuloza (polisaharid). U ovom radu je ispitana sposobnost ekspresije *curli* fimbrija i celuloze (preko morfotipa kolonija na Congo Red agaru) i produkcije biofilma (testom na mikrotitracionim pločama) kod 25 sojeva *E. coli* izolovanih iz mleka krava sa kliničkim mastitisom. Izolati su filogenetski grupisani na osnovu detekcije PCR produkata: *chuA*, *yjaA* i TspE4-C2. Osetljivost na antibiotike ispitana je standardnim disk difuzionim testom. Produkcija obe komponente ekstracelularnog matriksa ustanovljena je kod 56%, a ekspresija *curli* fibrija kod 64% izolata *E. coli*. Svi izolati koji su produkovali *curli* fimbrije su ovu osobinu ispoljili na temperaturi od

37°C, što ukazuje na mogući značaj ovih adhezivnih struktura u patogenezi mastitisa. Rezultati filogenetske tipizacije su potvrdili da su sojevi *E. coli* izolovani iz mleka krava sa mastitisom tipični komensali i da u najvećem procentu pripadaju filogenetskim grupama A i B1. Svi *curli* i *curli*/celuloza produkujući izolati formirali su biofilm *in vitro*. Biofilm može imati važnu ulogu u razvoju perzistentih infekcija, kao i recidivu kliničkih simptoma nakon antibiotskog tretmana, uprkos dobre osetljivosti uzročnika na antibiotike in vitro.