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Characterization of *Escherichia coli* Strains Derived from Cow Milk of Subclinical and Clinical Cases of Mastitis

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Abstract: Mastitis is a major economic problem in dairy herds, as it might decrease fertility, and negatively affect milk quality and milk yield. Out of over 150 bacterial species responsible for the udder inflammation, *Escherichia coli* is one of the most notable. This study aimed to assess antimicrobial susceptibility, resistance to dipping agents and biofilm formation of 150 *E. coli* strains isolated from milk of cows with subclinical and clinical mastitis. The strains came from three dairy herds located in Northern and Central Poland. The statistical analyses were performed with post-hoc Bonferroni test and chi-square test (including Yates correction). The data with a *p* value of <0.05 were considered significant. We found that the tested strains were mostly sensitive to antimicrobials and dipping agents. It was shown that 37.33% and 4.67% of strains were resistant and moderately resistant to at least one antimicrobial agent, respectively. No extended-spectrum beta-lactamases (ESBL)-producing *E. coli* were detected. The majority of strains did not possess the ability to form biofilm or formed a weak biofilm. The strong biofilm formers were found only among strains derived from cows with subclinical mastitis. The lowest bacteria number was noted for subclinical mastitis cows’ strains, after stabilization with iodine (3.77 log CFU cm⁻²) and chlorhexidine (3.96 log CFU cm⁻²) treatment. In the present study, no statistically significant differences in susceptibility to antibiotics and the ability to form biofilm were found among the strains isolated from cows with subclinical and clinical mastitis. Despite this, infections in dairy herds should be monitored. Limiting the spread of bacteria and characterizing the most common etiological factors would allow proper treatment.

Keywords: *Escherichia coli*; mastitis; milk cow; dairy cow; antimicrobial susceptibility; biofilm formation

1. Introduction

Bovine mastitis (BM) is one of the most important diseases in the dairy industry. Mastitis is an inflammation of the mammary gland causing chemical, physical, microbiological changes, and an increase in the number of somatic cells in the milk [1]. Two types of bovine mastitis are distinguished, i.e., clinical and subclinical. The clinical form contributes to pathological changes in the mammary tissue and physical, chemical, and usually bacteriological changes in the milk [2]. The subclinical form of mastitis is 15–40 times more prevalent and no obvious signs in the milk and udder are observed [3]. For subclinical mastitis, on-farm screening tests are used traditionally, such as somatic cell count (SCC), California mastitis test, and the Surf field mastitis test [4,5]. SCC is considered as the most popular indicator of mastitis detection and is widely used for determining milk quality in
individual quarters and bulk tanks. As required under The European Union Regulation 853/2004 [6], the SCC in raw milk must not exceed 400,000 cells/mL (based on a rolling geometric average over 3 months, with at least one sample per month). The United States has a limit of 500,000 cells/mL for consumption in the country [7]. The second step in mastitis control is the assessment of bacteriological aspects of the disease. The milk sample should be taken aseptically into a sterile container and transported to the laboratory as soon as possible. The disease might decrease fertility, negatively affect milk quality and milk yield, and lead to animal culling, thereby being the main reason for economic losses [8]. The major causes of mastitis are bacterial and fungal infections. The source of bacteria might be milkers’ hands, udder cloths, milking machines, and the environment (bedding, manure, and water). According to the European Food and Safety Authority (EFSA) report, the factors most frequently contributing to BM are insufficient hygiene of the litter and the milking machines [9]. Over 150 bacterial species responsible for udder inflammation were identified [10]. The pathogens most commonly isolated from cows with mastitis are coagulase-negative, coagulase-positive staphylococci, and environmental streptococci [11]. Additionally, E. coli accounts for many cases of bovine mastitis. E. coli is a Gram-negative, facultatively anaerobic, rod-shaped bacterium. E. coli is a natural resident of soil and cow’s intestine. The bacterium is also prevalent in manure, water, and bedding. E. coli is the most common cause of clinical mastitis in well-managed dairy herds, with a low number of somatic cells in the milk [12,13]. E. coli does not colonize the lactiferous gland but persists in the teat canal and mammary gland cistern. The intensification of clinical symptoms in acute mastitis is mostly associated with endotoxin LPS (lipopolysaccharide) [13]. An important therapeutic problem is the antibiotic resistance of E. coli. One of the resistance mechanisms in E. coli is the production of the enzyme extended-spectrum beta-lactamases (ESBL). ESBLs are known to hydrolyze all penicillins, early cephalosporins, oxyimino-cephalosporins, and monobactams, but lack any hydrolytic activity to cepamycins and carbapenems. ESBLs are inhibited by beta-lactamase inhibitors such as clavulanic acid, tazobactam, and sulbactam [14]. Our previous study [15] showed that teat cups, contaminated during milking might contribute to the transmission of bacteria to cow’s udders and finally to the milk. It was found that this phenomenon might affect at least several successively milked animals. This was also confirmed a study [16] that found that improper hygiene of teat cups could cause transmission of Staphylococcus spp. both between the animals and to the milk tank. One way to minimalize the risk of BM is an efficient teat predipping and postdipping, aiming to destroy the microorganisms on the udder skin and in a drop of the milk of the teat canal [17]. Dipping agents commonly applied for teat disinfection are iodine and its derivatives, and chlorhexidine. Another important aspect is the treatment of an infected animal with antibiotics. Such treatment, however, should consider the antimicrobial susceptibility of the pathogen. E. coli can be a reservoir of antibiotic resistance genes that might be transferred to other pathogenic bacteria [18,19]. An important skill allowing to survive in the unpropitious environment is a biofilm formation ability. Biofilm is a community of microorganisms encased in a self-formed matrix. Such structure increases bacterial resistance to antimicrobials and disinfectants enabling its persistence in the udder. Biofilm production is also regarded as an important virulence mechanism [11].

The present study aimed to assess antimicrobial susceptibility, resistance to dipping agents, and biofilm formation of E. coli strains isolated from milk of cows with clinical and subclinical mastitis.

2. Materials and Methods

2.1. Milk Sampling and Analyses

Three dairy herds in Kuyavian-Pomeranian and Pomeranian Voivodeship (located in North and Central Poland) were included in the study. The average dairy herd size was 87–123 cows, and the most common dairy cows’ breed was the Holstein. The samples were taken aseptically from all lactating cows, directly after milking between November 2007 and April 2015. Clinical mastitis diagnosis was performed by the veterinarian according to a
standardized procedure that included physical examination of the udder and the estimation of the somatic cells in the milk. Milk samples were examined for visible abnormalities and screened using the California mastitis test (CMT; scored 1 to 5 corresponding to no reaction, trace, mild reaction, moderate reaction, and strong reaction, respectively). Cows either with or without positive results in CMT and any clinical symptoms of other illness such as lameness or injuries were selected as cows with subclinical mastitis. Bacteriological analyses were done within 24 h after sampling. Among the samples from cows showing symptoms of mastitis, only those where \( E. coli \) was proved to be the etiological factor of the infection, were used in the experiment.

2.2. Bacterial Strains

The study was conducted on 150 \( E. coli \) strains, of which 109 and 41 were derived from the milk of cows with subclinical and clinical mastitis, respectively. All strains were identified using MALDI—TOF MS (Matrix-Assisted Laser Desorption Ionization Time of Flight, Mass Spectrometry), in accordance with the manufacturer procedure with MALDI Biotyper (Bruker, Billerica, MA, USA). The tested strains were stored at \(-80\) °C in BHI broth (Brain Heart Infusion, bioMérieux), with the addition of 15.0% glycerol (Avantor Performance Materials Poland S.A.).

2.3. Antimicrobial Susceptibility Testing and Detection of Extended-Spectrum Beta-Lactamases (ESBL)

Susceptibility of \( E. coli \) strains to the 31 selected antibiotics (listed in Table 1) was assessed on the Mueller-Hinton Agar (Becton Dickinson, Franklin Lakes, NJ, USA) with the Kirby-Bauer disk diffusion method, according to the recommendations of the European Committee on Antimicrobial Susceptibility Testing [20]. \( E. coli \) ATCC 25922 was used for quality-control purpose.

| Antibiotic | No. \( E. coli \) Subclinical (\( n = 109 \)) | p Value For The Resistant Strains of Both Groups |
|------------|-------------------------------------------|-----------------------------------------------|
|            | R (%) | I (%) | S (%) | R (%) | I (%) | S (%) |                          |
| AM         | 6 (5.5) | 0 (0.0) | 103 (94.5) | 2 (4.9) | 0 (0.0) | 39 (95.1) | 0.85 |
| SAM        | 1 (0.7) | 0 (0.0) | 108 (99.1) | 0 (0.0) | 0 (0.0) | 41 (100.0) | 0.40 |
| AMC        | 31 (28.4) | 0 (0.0) | 78 (71.6) | 10 (24.4) | 0 (0.0) | 31 (75.6) | 0.52 |
| PIP        | 6 (5.5) | 2 (1.8) | 101 (92.7) | 1 (2.4) | 2 (2.4) | 39 (95.1) | 0.26 |
| TZP        | 0 (0.0) | 2 (1.8) | 107 (98.2) | 0 (0.0) | 1 (2.4) | 40 (97.6) | 1.00 |
| TIC        | 13 (11.9) | 0 (0.0) | 96 (88.1) | 2 (7.3) | 0 (0.0) | 38 (92.7) | 0.29 |
| TIM        | 8 (7.3) | 0 (0.0) | 101 (92.7) | 2 (4.9) | 0 (0.0) | 39 (95.1) | 0.47 |
| CTX        | 0 (0.0) | 0 (0.0) | 109 (100.0) | 0 (0.0) | 1 (2.4) | 40 (97.6) | 0.12 |
| FOX        | 0 (0.0) | 0 (0.0) | 109 (100.0) | 0 (0.0) | 1 (2.4) | 40 (97.6) | 0.12 |
| CAZ        | 1 (0.9) | 4 (3.7) | 104 (95.4) | 0 (0.0) | 6 (14.6) | 35 (85.4) | 0.02 |
| CRO        | 0 (0.0) | 0 (0.0) | 109 (100.0) | 0 (0.0) | 0 (0.0) | 41 (100.0) | 1.00 |
| CTP        | 4 (3.7) | 0 (0.0) | 105 (96.3) | 1 (2.4) | 0 (0.0) | 40 (97.6) | 0.59 |
| CXM        | 0 (0.0) | 0 (0.0) | 109 (100.0) | 0 (0.0) | 0 (0.0) | 41 (100.0) | 1.00 |
| FEP        | 0 (0.0) | 1 (1.8) | 108 (98.1) | 0 (0.0) | 1 (2.4) | 40 (97.6) | 1.00 |
| DOR        | 0 (0.0) | 0 (0.0) | 109 (100.0) | 0 (0.0) | 0 (0.0) | 41 (100.0) | 0.00 |
| ETP        | 0 (0.0) | 0 (0.0) | 109 (100.0) | 0 (0.0) | 1 (2.4) | 40 (97.6) | 1.00 |
| IPM        | 0 (0.0) | 0 (0.0) | 109 (100.0) | 0 (0.0) | 1 (2.4) | 40 (97.6) | 1.00 |
| MEM        | 0 (0.0) | 0 (0.0) | 109 (100.0) | 0 (0.0) | 0 (0.0) | 41 (100.0) | 0.00 |
| ATM        | 2 (1.8) | 10 (9.2) | 97 (89.0) | 0 (0.0) | 1 (2.4) | 40 (97.6) | 0.18 |
| CIP        | 0 (0.0) | 6 (5.5) | 103 (94.5) | 2 (3.4) | 3 (7.3) | 38 (92.7) | 0.06 |
| LVX        | 2 (1.8) | 6 (5.5) | 101 (92.7) | 0 (0.0) | 4 (9.8) | 37 (90.2) | 0.53 |
| MXF        | 0 (0.0) | 0 (0.0) | 109 (100.0) | 0 (0.0) | 0 (0.0) | 41 (100.0) | 1.00 |
| NOR        | 7 (6.4) | 3 (2.8) | 99 (90.8) | 1 (2.4) | 4 (9.8) | 36 (87.8) | 0.17 |
| OFX        | 11 (10.1) | 1 (0.9) | 97 (89.0) | 4 (9.8) | 3 (8.0) | 33 (80.5) | 0.09 |
| AN         | 1 (0.9) | 0 (0.0) | 108 (99.1) | 0 (0.0) | 0 (0.0) | 41 (100.0) | 0.34 |
| Antibiotic | No. E. coli Subclinical (n = 109) | No. E. coli Clinical (n = 41) | p Value For The Resistant Strains of Both Groups |
|-----------|---------------------------------|-----------------------------|-----------------------------------------------|
|           | R (%) I (%) S (%)               | R (%) I (%) S (%)           |                                               |
| GM        | 0 (0.0) a 0 (0.0) 109 (100.0)   | 1 (1.7) a 0 (0.0) 41 (100.0) | 0.19                                          |
| NET       | 0 (0.0) a 0 (0.0) 109 (100.0)   | 0 (0.0) a 0 (0.0) 41 (100.0) | 1.00                                          |
| NN        | 0 (0.0) a 0 (0.0) 109 (100.0)   | 0 (0.0) a 0 (0.0) 41 (100.0) | 1.00                                          |
| TGC       | 0 (0.0) a 0 (0.0) 109 (100.0)   | 0 (0.0) a 1 (2.4) 40 (97.6)  | 1.00                                          |
| C         | 0 (0.0) a 0 (0.0) 109 (100.0)   | 0 (0.0) a 0 (0.0) 41 (100.0) | 1.00                                          |
| SXT       | 2 (1.8) a 0 (0.0) 107 (98.2)    | 1 (2.4) a 0 (0.0) 40 (97.6)  | 0.77                                          |

R—resistant, I—intermediate, S—susceptible, AM—ampicillin, SAM—ampicillin/sulbactam, AMC—amoxicillin/clavulnic acid, PIP—piperacillin, TZP—piperacillin/tazobactam TIC—ticarcillin, TIM-ticarcillin/clavulanic acid, CTX—cefotaxime, FOX—cefoxitin, CAZ—ceftazidime, CRO—ceftroxime, CFT—cetampicillin, FEP—ceftaxime, DOR—doripenem, ETP—ertapenem, IMI—imipenem, MEM—meropenem, ATN—aztreonam, CIP—ciprofloxacin, LVX—levofloxacin, MXF—moxifloxacin, NOR—norfloxacin, OFX—ofloxacin, AN-amikacin, GM—gentamicin, NET—netilmicin, NN—tobramycin, TGC—tigecycline, C—chloramphenicol, SXT—trimethoprim/sulfamethoxazole; a,b—values marked with different letters in the same row in column for resistance differ statistically significantly (p ≤ 0.05) for a particular antibiotic.

Production of ESBL was assessed using the double-disk synergy test [21]. E. coli ATCC 25922 (not producing ESBLs) and Klebsiella pneumoniae ATCC 700603 (producing ESBLs) were used as the reference strains.

2.4. Evaluation of the Effectiveness of Dipping Agents

The efficacy of dipping substances was assessed on 30 E. coli strains of cows with a clinical form of mastitis and 30 E. coli strains of cows with subclinical mastitis. The evaluation of effectiveness of dipping agents was previously described by Skowron et al. [17]. Fragments of the teat skin of slaughtered cows were used. The slaughter was not an element of the study. The skin deprived of subcutaneous fat was cut to fragments of 1 cm × 1 cm. The fragments were immersed in 70% ethanol for 5 min, and air-dried in a sterile laminar chamber. Next, each side of the fragment was exposed to UV light for 20 min. Such fragments were packed and stored at −20 °C, until use. The tested dipping agents were—stabilized iodine, povidone iodine (PVP), and chlorhexidine. Fragments of teat skin were soaked in suspensions of all examined strains (one strain per one fragment) and left to dry. For each strain, a suspension of 0.5 McFarland’s scale [5.20 × 10^8 (±2.86 × 10^7)] was prepared. The contaminated fragments were immersed for 20 min in the examined dipping agents (one agent per one fragment), then were shaken for 2 min (400 rpm) in the neutralizing medium (tween 80—10.0 g; lecithin—1.0 g; histidine L—0.5 g; Na_2S_2O_3—2.5 g; water—1000 mL) and subjected to sonication (20 min). After sonication, serial 10-fold dilutions in sterile saline were prepared and plated onto an appropriate culture medium (0.1 mL) (Figure 1).

After incubation (37 °C, 24 h), bacterial colonies were counted and the percentage of reduction (R) bacteria number, as compared to the positive control, was determined using the following formula:

\[
R = \frac{K(+) - A}{K(+) } \times 100
\]

where K(+)—the number of bacteria from the control sample [log_{10} CFU × cm^{-2}]; and A—the number of bacteria of a given strain [log_{10} CFU × cm^{-2}].

For each strain, the experiment was conducted in three replicates. The negative control was a fragment of skin not submerged in the bacterial suspension.
2.5. Evaluation of Biofilm Formation

Biofilm formation ability was assessed in the group of 30 *E. coli* strains derived from cows with subclinical mastitis and 30 strains isolated from cows with clinical mastitis, using the crystal violet method [16]. The studied strains were plated on Trypticase soy agar (TSA) (bioMérieux) and incubated at 37 °C for 24 h. Next, bacterial suspensions in Trypticase soy broth (TSB) (0.5 McF) (bioMérieux) were prepared and 200 µL of each suspension was placed in 96-well plates, in triplicates, for each strain. *Staphylococcus aureus* ATCC 6538P (very strong biofilm-forming strain) and *E. coli* ATCC 35218 (weak biofilm-forming strain) reference strains were used as controls. A negative control (200 µL of TSB medium) was made in at least four repetitions. Plates were incubated at 37 °C for 24 h in a humid chamber. After 24-incubation, the suspensions were removed, the wells were rinsed with sterile distilled water and left to dry at 37 °C for 20 min. Next, 200 µL of pure methanol (Avantor Performance Materials Poland S.A.) was added to each well. Subsequently, the plates were shaken for 20 min (400 rpm), methanol was removed and the plates were dried at 37 °C for 20 min. Afterward, 200 µL of 0.1% crystal violet (CV) was pipetted to each well and the plates were shaken (400 rpm, 20 min). Then, the dye was removed and the wells were washed with distilled water to obtain colorless washings. The plates were left for 20 min at 37 °C to allow the evaporation of the remaining water. Eventually, 200 µL of pure methanol was added to each well, plates were shaken (400 rpm, 5 min), and the absorbance (A) at 570 nm was measured (The Synergy HT Multidetection) (Figure 2).
Subsequently, the threshold value of absorbance (T), defined as the sum of the arithmetic mean of negative control and a triple value of its standard deviation (T = xnc + 3δ), was established. A value below the calculated sum was recognized as weak biofilm. Mild biofilm was determined when the value of sum was between T and 2T, moderate biofilm—between 2T and 4T, and strong for a value higher than 4T [22].

2.6. Statistical Analysis

Statistical analysis was carried out using Statistica 13.1 PL software (StatSoft, Crakow, Polska). The significant differences in the frequency of resistance to the tested antibiotics, the intensity of biofilm formation, and the percentage reduction after the use of dipping agents between the subclinical and clinical *E. coli* strains were assessed using the chi-square test and the Fisher exact test, at the significance level $\alpha = 0.05$.

The number of bacteria isolated after the use of dipping agent was averaged separately for both groups of strains and compared with each other, using the analysis of variance ANOVA and the post-hoc Bonferroni test, at the significance level $\alpha = 0.05$. The dipping agent and a group of *E. coli* strains were treated as independent variables, and the number of recovered bacteria was treated as a dependent variable.
3. Results

A total of 2460 quarters from 360 cows in three dairy herds were analyzed in this study. A total of 1920 quarters were cultured for *E. coli* isolation.

3.1. Antibiotic Susceptibility Analysis

Eighty-seven strains (58.0%) were sensitive to all tested antibiotics. Among 150 strains, 56 (37.33%) were resistant and 7 (4.67%) intermediate resistant to at least one antimicrobial agent. None of the tested strains was resistant to all antimicrobials used. Resistance to amoxicillin with clavulanic acid (27.3%), ticarcillin (10.7%), ofloxacin (10.0%), norfloxacin, and ampicillin (5.3%) were the most prevalent. All strains were susceptible to cefuroxime, ceftriaxone, doripenem, meropenem, gentamycin, netilmicin, tobramycin, moxifloxacin, and chloramphenicol (Table 1). There were no significant differences in antimicrobial resistance between strains from cows with subclinical mastitis and isolates derived from clinical mastitis cases. The exception was resistance to ceftazidime (CAZ), which was statistically significant (*p* = 0.02). None of the tested *E. coli* strains produced extended-spectrum beta-lactamases (ESBL).

3.2. Assessment of Biofilm Formation

The tested *E. coli* strains formed biofilm with a different intensity. However, there was no difference in biofilm formation between strains derived from cows with subclinical and clinical mastitis. In both groups, over 80% of isolates formed weak biofilm or did not possess such ability at all. Strong biofilm formers were found only in the strains from cows with subclinical mastitis (*n* = 2), whereas the moderate ability of biofilm formation was noted in 3 and 6 strains, isolated from the subclinical and clinical cases of mastitis, respectively (Table 2).

### Table 2. The intensity of *E. coli* biofilm formation depending on the strains origin.

| The Intensity of Biofilm Formation | Number of Strains |  |
|---|---|---|
|  | *E. coli* Subclinical (*n* = 30) | *E. coli* Clinical (*n* = 30) |
| Lack | 11 (36.7) | 11 (36.7) |
| Weak | 14 (46.7) | 13 (43.3) |
| Moderate | 3 (10.0) | 6 (20.0) |
| Strong | 2 (6.6) | 0 (0.0) |

1—number of strains, 2—percentage of strains; and 3ab—values marked with different letters are statistically significant (*p* ≤ 0.05).

3.3. Evaluation of Resistance to Dipping Agents

The number of bacteria reisolated from skin fragments not treated with dipping substances ranged from 5.57 log CFU × cm⁻² (strains isolated from subclinical mastitis) to 6.02 log CFU × cm⁻² (strains derived from cows with clinical mastitis). The application of dipping agents significantly (*p* ≤ 0.05) reduced the number of bacteria reisolated from the udder surface (Figure 1). Stabilized iodine and chlorhexidine treatment decreased the number of subclinical mastitis cows’ strains most efficiently (3.77 log CFU × cm⁻² and 3.96 log CFU × cm⁻², respectively). Strains derived from clinical mastitis cases were more resistant, with the highest bacteria number for stabilized iodine (4.42 log CFU × cm⁻²) and PVP iodine (4.42 log CFU × cm⁻²) (Figure 3).
A rate expressed in percentage ranged from 96.39% for PVP iodine (subclinical strains) to 97.92% for stabilized iodine (subclinical strains) (Table 3). The differences were not statistically significant.

Table 3. Percentage reduction [R%] in the bacteria number.

| Type of Dipping Agent | Number of Strains | \( p \) Value (chi-Square Test) | Total (\( n = 60 \)) |
|-----------------------|-------------------|-------------------------------|---------------------|
|                       | \( E. \text{coli} \) Subclinical (\( n = 30 \)) | \( E. \text{coli} \) Clinical (\( n = 30 \)) |                       |
| Povidone iodine       | 96.39 \( \text{a} \) | 96.23 \( \text{a} \) | \( p = 0.95 \) | 22 (36.7) |
| Stabilized iodine     | 97.92 \( \text{a} \) | 96.82 \( \text{a} \) | \( p = 0.63 \) | 27 (45.0) |
| Chlorhexidine         | 96.19 \( \text{a} \) | 96.96 \( \text{a} \) | \( p = 0.76 \) | 9 (15.0) |

\( \text{a} \)—values marked with different letters are statistically significant (\( p \leq 0.05 \)).

4. Discussion

Mastitis is the most prevalent disease and the main economic problem in dairy herds worldwide [23]. It is estimated that each udder inflammation case generates a loss of 200 Euro per cow [24]. Annually, in the United States, dairy farmers lose approximately 2 billion dollars because of BM [11]. Therefore, it is crucial to eliminate possible pathogens’ transmission routes and minimize the risk of infection. On the other hand, all cases of mastitis require recording and treatment with appropriate antimicrobials. The overuse of antimicrobials in animal husbandry has led to the selection of antibiotic-resistant bacteria that can readily disseminate in the environment. Therefore, the antibiotic resistance profile of the pathogen is indispensable for successful treatment. The main source of antimicrobial resistance (AMR) bacteria is the use of antimicrobials in human medicine, especially in hospitals [25]. An important factor contributing to AMR is also the use of antibiotics in animal production that might have long-term consequences. Farming animals might spread antibiotic resistant bacteria to other animals, including wild and domestic ones. Direct contact with such animals, as well as consumption of their meat or milk, pose a risk to humans [26]. One of the most notable bacteria responsible for bovine mastitis are E. coli. The first-line
drug for BM caused by E. coli is gentamicin and fourth-generation cephalosporins are recommended as second-line therapy [27]. However, the fourth-generation cephalosporins are the highest priority critically important antimicrobials for use in humans, mainly due to the high risk of dissemination of extended-spectrum β-lactamase (ESBL) isolates in Enterobacterales species [28].

In the present study, the characterization of E. coli strains isolated from cows with subclinical and clinical mastitis were investigated. Almost 40% of all tested strains (n=56) were resistant to at least one antimicrobial agent. There were no significant differences in antibiotic susceptibility between strains of subclinical mastitis and strains derived from clinical mastitis. One exception shown was resistance to ceftazidime. The most common in both groups was resistance to amoxicillin with clavulanic acid. All strains were sensitive to cefuroxime, ceftriaxone, doripenem, meropenem, gentamicin, netilmicin, tobramycin, moxifloxacin, and chloramphenicol. None of the strains produced ESBL. Similarly, ESBL producers are described infrequently in E. coli isolated from bovine mastitis in Germany, France, Switzerland, and the UK [29–32]. The susceptibility of E. coli strains isolated from milk samples is also documented by Hinthong et al. [18] and Ameen et al. [33]. The majority (68%) of the E. coli isolates from cows with mastitis in Switzerland were fully susceptible to all antimicrobials tested. The authors observed low resistance rates for gentamicin (3.7%), amoxicillin/clavulanic acid (2.4%), and ceftiofur (1.2%) [34]. The low resistance rates were also reported among mastitis E. coli isolates in Denmark with no resistance to gentamicin and ceftiofur [35]. On the other hand, Hinthong et al. [18] observed resistance to cefuroxime, ceftriaxone, gentamicin, and the ESBL phenotype in 23% of isolates derived from the milk of cows with subclinical mastitis.

One of the most efficient ways of mastitis case reduction in dairy farms is pre-milking and post-milking teat disinfection. In the present study, antibacterial activity of three dipping agents, i.e., stabilized iodine, povidone iodine, and chlorhexidine was evaluated. All three agents significantly reduced the bacteria number (from 96.16 to 97.92%). The strain susceptibility was not correlated with its origin. There were no significant differences in the bactericidal efficacy between the dipping agents. This supports our previous study showing the high effectiveness of all three chemicals against E. coli, S. aureus, L. monocytogenes, and S. marcescens [17]. Additionally, Pelletier et al. [36] showed high effectiveness of preparations based on iodine at various concentrations in teat dip.

One property helping the bacteria to survive in the mammary gland and persist in dairy herds is a biofilm formation ability [11]. Biofilm production might hinder the treatment of recurrent infection. In this study, we compared this ability between a group of strains isolated from cows with subclinical and clinical mastitis. To date, no significant link between biofilm formation capability and bovine mastitis was demonstrated [37]. In our study, we did not observe a significant difference in the biofilm intensity between both groups of isolates. On the contrary, Fernandes et al. [38] reported that all 27 E. coli strains isolated from cows with mastitis possessed the capability to form biofilm, although with different strength. Dubravka et al. [39], however, demonstrated that biofilm production is strictly associated with the medium and growth conditions applied. They revealed that lower temperature (20 °C) and low nutrient media promoted the biofilm formation ability of E. coli mastitis’ strains. In turn, cultivation in TSB at 37 °C inhibited this property in almost 80% of the tested strains [39].

5. Conclusions

This study aimed to characterize E. coli strains from the milk of cows with clinical and subclinical mastitis. The majority of strains were sensitive to all tested antibiotics. No ESBL-producing E. coli were detected. The majority of strains did not possess the ability to form biofilm or formed weak biofilm. The difference in the ability to form biofilm was not statistically significant between both groups. All dipping agents used in the study exhibited very high efficacy against E. coli strains. The most effective dipping agent was stabilized iodine against E. coli strains from subclinical mastitis. Collectively, the mastitis...
strains did not possess greater AMR potential, biofilm formation ability, and resistance to dipping agents than the subclinical strains.

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