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# Potential Risk of Antimicrobial Resistance Related to Less Common Bacteria Causing Subclinical Mastitis in Cows

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## Abstract

Antimicrobials are an essential tool for intra-mammary infection control. This study was achieved to assess the resistance to antimicrobials as a risk associated with less common bacteria identified in subclinical mastitis (SCM) milk samples of dairy cows. The disc diffusion method was used for determining the resistance to antimicrobials. The interrelate resistance genes were also detected by polymerase chain reaction (PCR). The antimicrobial sensitivity test indicated that ampicillin, oxacillin, gentamicin, tetracycline, amoxicillin + clavulanic acid, oxytetracycline, and cephradine were highly resistant antibiotics against gram-positive bacilli microorganisms. However, the highest effective antibiotic against the investigated gram-negative bacilli isolates was gentamicin. The antimicrobial resistance genes investigation showed that the *tetA(A)* and *BlaTEM* genes were expressed in all the Gram-negative bacilli isolates. The *MecA* and *blaZ* were positive in the investigated *Staphylococcus chromogenes* isolates, while all *B. cereus* and *B. subtilis* isolates were positive for the *Bla* gene. The *Sul1* gene was positive in all the examined *Citrobacter amalanaticus*, *Enterobacter* species, and 50.0% of *Klebsiella oxytoca* isolates. The mph (A) gene was found in all *Enterobacter* species isolates.

KEYWORDS

Resistance to antimicrobials, Subclinical mastitis milk, Gram-negative bacilli, *tetA(A)* gene, *BlaTEM* gene

## INTRODUCTION

The most precious resource that boosts a nation's economy and raises community living standards are dairy cows (Verma *et al*., 2018). Subclinical mastitis (SCM) is an inflammation of the milk glands without the presence of obvious lesions in the udder. It is more prevalent (fifteen to forty times) than the observed type, reduces milk production, which causes the agriculturalist to have a budget gap, and increases the risk of contracting a clinical infection. Moreover, the milk is produced with no noticeable alterations and can be mixed into bulk milk, which may pose a health threat to humans. Therefore, the detection of SCM is not achieved without continuous monitoring (Leskovec *et al*., 2015; Youssif *et al*., 2021a).

The translocation of bacteria from the environment to the end of the teat is the first stage in the formation of SCM infection. For infectious germs, the transfer takes place during the milking procedure. The milker's hands, udder cloths, and milking machine are incriminated for transferring the infection from the infected to the uninfected cow's udder quarter (Almaw *et al*., 2012). Due to its complicated etiology, SCM is challenging to manage. Three important elements that may be related to the number of bacteria acting as a contributing agent, its drug resistance, its toxin content, or related management and environmental conditions can impact it. Cleanliness and sanitary environments are significant factors in limiting illness and lowering the likelihood of pathogen survival (Darbaz *et al*., 2018).

According to Ruelle *et al*. (2019); antimicrobials are frequently used to treat dairy cows with SCM. Additionally, there is a lack of strict regulation on the use of antibiotics in animal management systems. If SCM is not monitored and treated, fibrous tissue may form between the germs and the antimicrobial drugs, thus reducing their efficacy and possibly causing bacterial antibiotic resistance.

Antibiotics are significant agents for the control and prevention of widespread diseases in the dairy sector. Newly, there is significant consideration concerned with antimicrobial residues in milk that leads to the expansion of resistance genes and moving between human and animal microorganisms (Schewe and Brock, 2018). Humans, animals, food, and the environment are significant sources of resistant pathogens to antimicrobials, which can disseminate between human and animals and from one individual to another. Inefficient infection control, insufficient hygienic surroundings, and unsuitable food control may accelerate the expansion of AMR in the community (Hay *et al*., 2018).

Antimicrobial Resistance (AMR) is a problem concerning public and animal health. However, antimicrobials are one of the tools for controlling udder inflammation. The intensive misuse of antimicrobials can cause antibiotic residues in milk, activating the risk of bacteria establishing AMR in milk or milk product consumers. Also, have a great concern about the spreading of Multi-Drug Resistant (MDR) microorganisms, which express resistance


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to at least one antimicrobial drug in three or more antimicrobial categories (Zdolec *et al*., 2013; Ombarak *et al*., 2019). Therefore, the objective of this study was to evaluate the antimicrobial resistance levels for the less frequent bacterial species causing bovine SCM and their related resistance genes.

## MATERIALS AND METHODS

*Area of study and cows*

Six hundred and ninety-five lactating cows from a total of 2300 Holstein Friesian dairy cows in a particular dairy farm in Fayoum, Egypt, that used the Afi, Milking Point Controller system were chosen for this study (one visit/week). Milk yield and milk conductivity were measured by Milking Point Controller for monitoring milk output and animal health. SCM could detect by elevating Electrical conductivity (EC) which, is accompanied by a decrease in milk output and gives an alarm that allows the milkman to concentrate precisely on the particular cows. Four hundred forty-four lactating cows were positive for SCM by California Mastitis Test (CMT) based on Schalm *et al*. (1971).

*Cow's milk samples collection and examination*

Four hundred forty-four SCM cow milk samples were collected aseptically; teat ends were dipped into an antiseptic solution (iodine 1.0%) and dried. The first milk flow was rejected to minimize the bacterial load in the teat canal and followed by visual examination for any abnormalities detection as reported by Nesma *et al*. (2020) and prepared for bacterial investigation based on Carter and Cole (2012). The MacConkey agar (Oxoid, CM0115), Pseudomonas agar (Oxoid, CM0559), Bacillus-specific agar (Oxoid, CM0617), and Mannitol salt agar plates (Oxoid, CM0085) was utilized for bacterial isolation. The nutrient slope agar (Lab M, LAB008) was inoculated with suspected colonies and kept at 37°C/48 h for subsequent identification. The following techniques were used for colonies identification; colony characteristics, Gram staining, motility test, Haemolytic classification, Tube Coagulase test, Thermostable Nuclease test, and various biochemical examinations (Oxidase test, Nitrate reduction test, Indole test, Methyl Red test, Voges- Proskauer test, Citrate utilization, $H_2S$ production, Urea test, Sugars fermentation according to Whitman *et al*. (2015), and the Vitek2 compact system (BioMérieux, France).

*Ethical approval*

The study was approved by the ethical committee of the Faculty of Veterinary Medicine, Cairo University as a portion of the Ph.D., dissertation.

*The antimicrobial resistance level of isolates by disk diffusion method (CLSI, 2018)*

Isolated colonies were suspended in tryptone soya broth (Oxoid- CM0129) using a sterile loop and incubated at 37 °C for 18 h. Turbidity was matched to the standard McFarland tube No 0.5% ($1.5\times10^8$cell/ml). Using a swab that was immersed in the standard suspension, the Muller Hinton agar plate (Oxoid- CM0405B) was streaked in different planes for even distribution and kept on a flat surface for 5-10 minutes to allow the adsorption of excess fluid. By sterile fine-pointed forceps, selected antibiotic discs were placed on the inoculated plates with gentle pressure onto the agar to ensure complete contact with the surface. The plates were preserved at 37°C for 24 h. Zones (including the diameter of the disc) were determined to the adjacent whole millimeter by a ruler.

The antibiotic discs and their concentration (IU for penicillin or µg for others) were used like the following; (Penicillin- Norfloxacin- Gentamicin and Ampicillin): 10 for each, (Enrofloxacin- Epicoflosin and Rifampicin): 5 for each, (Tetracycline- Doxycycline- Amikacin- Oxytetracycline- Amoxacillin+ clavulanic acid- Cephradine- Tetracycline- Cefoxitin- Chloramphenicol, and Kanamycin): 30 for each, Sulfamethoxazole/ Trimethoprim (S/T): 25, Nitrofurantoin: 300, Oxacillin: 1, Erythromycin:15, and Clinda-

Table 1. Sequence of used primer for each investigated gene.

| Gene | Primer Sequence (5'-3') F: forward primer R: reverse primer | Product of PCR | Reference |
|---|---|---|---|
| *tetA(A)* | F: GGTTCACTCGAACGACGTCA<br>R: CTGTCCGACAAGTTGCATGA | 576 base pairs | Randall *et al*. (2004) |
| *MecI* | F: GACACGTGAAGGCTATGATATAT<br>R: ATTCTTCAATATCATCTTCGGAC | 344 base pairs | Stegger *et al*. (2012) |
| *bla* | F: CATTGCAAGTTGAAGCGAAA<br>R: TGTCCCGTAACTTCCAGCTC | 680 base pairs | Chen *et al*. (2004) |
| *blaZ* | F: ACTTCAACACCTGCTGCTTTC<br>R: TGACCACTTTTATCAGCAACC | 173 base pairs | Duran *et al*. (2012) |
| *MecC* | F: GCTCCTAATGCTAATGCA<br>R: TAAGCAATAATGACTACC | 304 base pairs | Cuny *et al*. (2011) |
| *Sul1* | F: CGGCGTGGGCTACCTGAACG<br>R: GCCGATCGCGTGAAGTTCCG | 433 base pairs | Ibekwe *et al*. (2011) |
| *MecA* | F: GTAGAAATGACTGAACGTCCGATAA<br>R: CCAATTCCACATTGTTTCGGTCTAA | 310 base pairs | McClure *et al*. (2006) |
| *Mph(A)* | F: GTGAGGAGGAGCTTCGCGAG<br>R: TGCCGCAGGACTCGGAGGTC | 403 base pairs | Nguyen *et al*. (2009) |
| *QnrS* | F: ACGACATTCGTCAACTGCAA<br>R: TAAATTGGCACCCTGTAGGC | 417 base pairs | Robicsek *et al*. (2006) |
| *QnrA* | F: ATTTCTCACGCCAGGATTTG<br>R: GATCGGCAAAGGTTAGGTCA | 516 base pairs | |
| *BlaTEM* | F: ATCAGCAATAAACCAGC<br>R: CCCCGAAGAACGTTTTC | 516 base pairs | Colom *et al*. (2003) |

mycin: 2.

*Resistance genes detection by Polymerase Chain Reaction (PCR)*

QIAamp DNA Mini Kit (Qiagen, Germany) was utilized for the extraction of Deoxyribonucleic acid with the manufacturer's directives. Absolute ethyl alcohol (Applichem, Darmstadt, Germany) was applied for the 1st step of the wash. The PCR master mix was the Emerald Amp GT PCR master mix (Takara, BIO INC., Japan, and code no. RR310A). A dye (vivid green color) was detached into yellow and blue, on an agarose gel. Next PCR, the mixture was achieved immediately to a gel for investigation.

Primers were obtained from Metabion (Germany). The used positive control for *MecA* was ATCC 43300. For other genes, positive and or negative controls were depicted by field samples, which were previously ascertained to be positive or negative by PCR for the associated genes in the reference experimenter for quality control, Animal health research institute, Cairo, Egypt. The status of PCR was primary denaturation at 94˚C/5 min and secondary denaturation at 94˚C/ thirty s. Annealing at 50˚C/30 s for all the examined genes except for *tetA(A)* and *BlaTEM* at 53˚C/ thirty s. The extension was at 72˚C/ thirty s. The number of cycles was thirty-five, and the final extension was at 72˚C/ seven min. Table 1 contained their appointed sequences and amplified products.

*Statistical analysis*

Statistical manipulation of data was done by using Microsoft Excel 365 enterprise (Microsoft, United States).

## RESULTS

The bacteria that were isolated from the 444 SCM cow's milk samples were *S. chromogenes*, *K. oxytoca*, *B. subtilis*, *P. aeruginosa*, *B. cereus*, *Enterobacter* species, *Sphingomonas paucimobilis*, *Raoultella ornithinolytica*, and *Citrobacter amalanaticus* with a percentage of 17.34, 13.73, 10.13, 7.20, 6.08, 4.05, 3.82, 2.70, and 0.67, respectively. The selected ten *S. chromogenes* isolates were 50.0% resistant to penicillin. However, all (100.0%) examined isolates were sensitive to oxacillin, gentamicin, clindamycin, erythromycin, tetracycline, and nitrofurantoin. All the examined *B. cereus* isolates were resistant to ampicillin, oxacillin, gentamicin, Sulfamethoxazole/ trimethoprim, amikacin, amoxicillin + clavulanic acid, oxytetracycline, and cephradine. On the other hand, ten examined Bacillus subtilus isolates expressed 100.0% resistance against ampicillin, oxacillin, gentamicin, tetracycline, amoxicillin + clavulanic acid, oxytetracycline, cefotaxime, doxycycline, and cephradine (Table 2).

*Klebsiella oxytoca* isolates were resistant (100.0%) to ampicillin, oxacillin, clindamycin, erythromycin, tetracycline, trimethoprim/ sulfamethoxazole, penicillin, and amoxacillin+ clavulanic acid. The *Sphingomonas paucimobilis* isolates were resistant (100.0%) to antibiotics (ampicillin, oxacillin, amikacin, and penicillin).

All *Raoultella ornithinolytica* isolates were resistant to ampicillin, oxacillin, clindamycin, tetracycline, and penicillin, while the ten examined *Pseudomonas aeruginosa* isolates expressed resistance 100.0% to cefoxitin, tetracycline, trimethoprim/ Sulfamethoxazole, penicillin, and amoxacillin+ clavulanic acid. The three examined *C. amalanaticus* were 100.0% resistant to tetracycline, trimethoprim/ sulfamethoxazole, penicillin, and amoxacillin+ clavulanic acid, as well as cefoxitin showed also 100.0% resistance to *Enterobacter* species isolates (Table 3).

The *blaZ* and *MecA* genes were found in the examined *Staphylococcus chromogenes* isolate, while the mec1 and *MecC* genes could not be detected in the examined *Staphylococcus chromogenes* (Fig. 1). The results recorded in Table 4 and Figs. 2- 8, revealed that the *B. cereus* and *B. subtilis* were positive for the *bla* gene, while the examined isolates of *Klebsiella oxytoca*, *Sphingomonas paucimobilis*, *Citrobacter amalanaticus*, *Enterobacter* species, *P. aeruginosa*, and *R. ornithinolytica* were positive for *tetA(A)* and *BlaTEM* genes. The *Sul1* gene was found in isolates of *C. amalanaticus*, *Enterobacter* species, and *K. oxytoca* in a percentage of 100, 100, and 50. In addition to *mph(A)* gene was detected in the *Enterobacter* species.

Table 2. Antimicrobial sensitivity test for Gram + ve bacilli microorganisms.

| Gram + ve bacilli | *Bacillus cereus* n.=10 | | *Bacillus subtilus* n.=10 | |
|---|---|---|---|---|
| Agent | S | R | S | R |
| Ampicillin | 0 | 100 | 0 | 100 |
| Oxacillin | 0 | 100 | 0 | 100 |
| Gentamicin | 0 | 100 | 0 | 100 |
| Tetracycline | 100 | 0 | 0 | 100 |
| Nitrofurantoin | 100 | 0 | 100 | 0 |
| Sulfamethoxazole/Trimethoprim | 0 | 100 | 50 | 50 |
| Amikacin | 0 | 100 | 100 | 0 |
| Penicillin | 100 | 0 | 50 | 50 |
| Enrofloxacin | 100 | 0 | 100 | 0 |
| Amoxacillin+ clavulanic acid | 0 | 100 | 0 | 100 |
| Norfloxacin | 100 | 0 | 50 | 50 |
| Epicoflosin | 100 | 0 | 100 | 0 |
| Oxytetracycline | 0 | 100 | 0 | 100 |
| Cefotaxime | 100 | 0 | 0 | 100 |
| Doxycycline | 100 | 0 | 0 | 100 |
| Kanamycin | 100 | 0 | 100 | 0 |
| Cephradine | 0 | 100 | 0 | 100 |

Values equal % S=sensitive R=resistant n. = Number of examined isolates.

Table 3. Antimicrobial sensitivity tests result of the examined Gram –ve bacilli microorganisms.

| Bacteria | *Klebsiella oxytoca* n.= 20 | | *Sphingomonas pauci-mobilis* n.= 10 | | *Raoultella ornithino-lytica* n.= 6 | | *Citrobacter amalanaticus* n. = 3 | | *Enterobacter* species n. = 5 | | *Pseudomonas aerugi-nosa* n. = 10 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Agent | S | R | S | R | S | R | S | R | S | R | S | R |
| Cefoxitin | 50 | 50 | 50 | 50 | 100 | 0 | 33.3 | 66.6 | 0 | 100 | 0 | 100 |
| Ampicillin | 0 | 100 | 0 | 100 | 0 | 100 | - | - | - | - | - | - |
| Oxacillin | 0 | 100 | 0 | 100 | 0 | 100 | - | - | - | - | - | - |
| Gentamicin | 50 | 50 | 50 | 50 | 100 | 0 | 100 | 0 | 100 | 0 | 100 | 0 |
| Clindamycin | 0 | 100 | - | - | 0 | 100 | 66.6 | 33.3 | 80 | 20 | - | - |
| Erythromycin | 0 | 100 | - | - | - | - | - | - | - | - | - | - |
| Tetracycline | 0 | 100 | 30 | 70 | 0 | 100 | 0 | 100 | 0 | 100 | 0 | 100 |
| Nitrofurantoin | - | - | 100 | 0 | - | - | - | - | - | - | 100 | 0 |
| S/T | 0 | 100 | 100 | 0 | - | - | 0 | 100 | 0 | 100 | 0 | 100 |
| Amikacin | 100 | 0 | 0 | 100 | 100 | 0 | - | - | 80 | 20 | 100 | 0 |
| Penicillin | 0 | 100 | 0 | 100 | 0 | 100 | 0 | 100 | 0 | 100 | 0 | 100 |
| Enrofloxacin | 100 | 0 | 100 | 0 | 100 | 0 | 33.3 | 66.6 | 80 | 20 | 100 | 0 |
| Amoxacillin+ clavulanic acid | 0 | 100 | 50 | 50 | 100 | 0 | 0 | 100 | 0 | 100 | 0 | 100 |
| Norfloxacin | 100 | 0 | 100 | 0 | 0 | 0 | 100 | 0 | - | - | 100 | 0 |

Values equal % S=sensitive R=resistant - = not detected n. = Number of examined isolates.

Table 4 Antimicrobial resistance genes for the examined Gram – ve and Gram + ve bacilli microorganisms.

| | *Klebsiella oxytoca* | *Sphingomonas Paucimobilis* | *Citrobacter amalanaticus* | *Enterobacter* species | *Pseudomonas aeruginosa* | *Raoultella ornithinolytica* | *Bacillus cereus* | *Bacillus subtilis* |
|---|---|---|---|---|---|---|---|---|
| *tetA(A)* | 100 | 100 | 100 | 100 | 100 | 100 | 0 | 0 |
| *BlaTEM* | 100 | 100 | 100 | 100 | 100 | 100 | 0 | 0 |
| *Sul1* | 50 | 0 | 100 | 100 | 0 | 0 | 0 | 0 |
| *Bla* | 0 | 0 | 0 | 0 | 0 | 0 | 100 | 100 |
| *Mph(A)* | 0 | 0 | 0 | 100 | 0 | 0 | 0 | 0 |

Values equal %

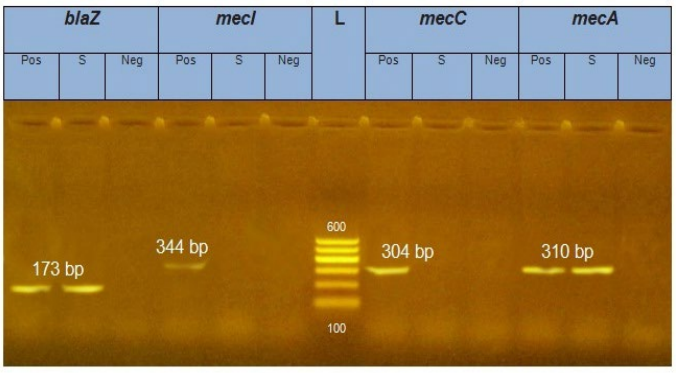


Fig. 1. Electrophoresis of detected antimicrobial resistance gene deoxyribonucleic acid fragments of *S. chromogenes* amplified by PCR from strains (*blaZ*- 173bp), (*MecA* – 310bp), (mec1- 344bp) and (*MecC*- 304bp).

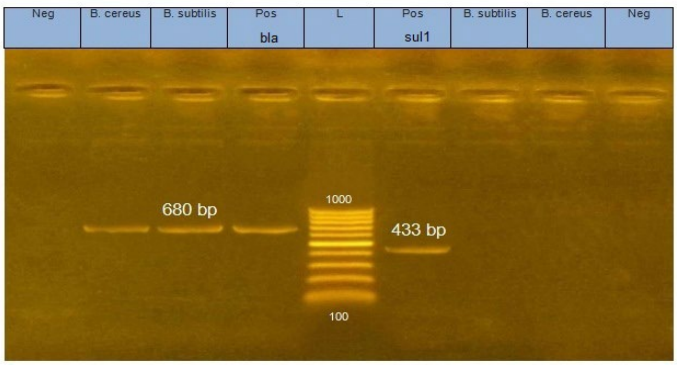


Fig. 2. Electrophoresis of detected antimicrobial resistance gene deoxyribonucleic acid fragments of Bacillus species amplified by (PCR) from strains (*bla*- 680bp) and (*Sul1* – 433bp).

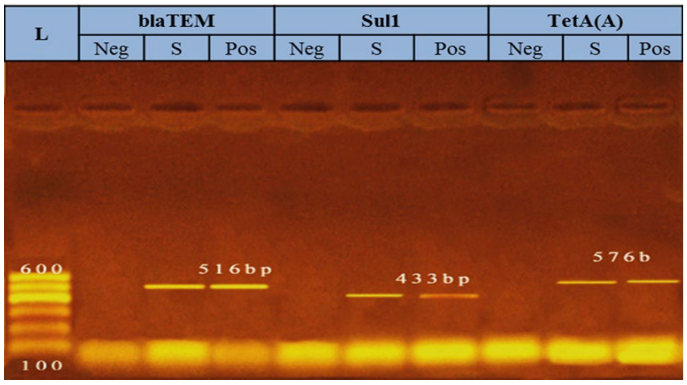


Fig. 3. Electrophoresis of detected antimicrobial resistance gene deoxyribonucleic acid fragments of *C. amalanaticus* amplified by (PCR) from strains (*tetA(A)* – 576 bp), (*BlaTEM*-516bp), and (*Sul1* – 433bp).

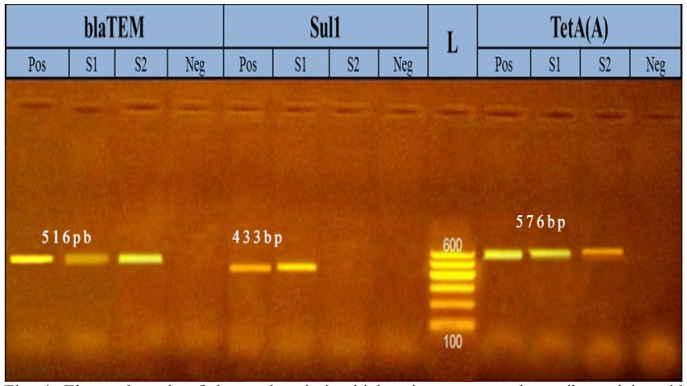


Fig. 4. Electrophoresis of detected antimicrobial resistance gene deoxyribonucleic acid fragments of *K. oxytoca* amplified by (PCR) from strains (*tetA(A)* – 576 bp), (*BlaTEM*-516bp) and (*Sul1* – 433bp).

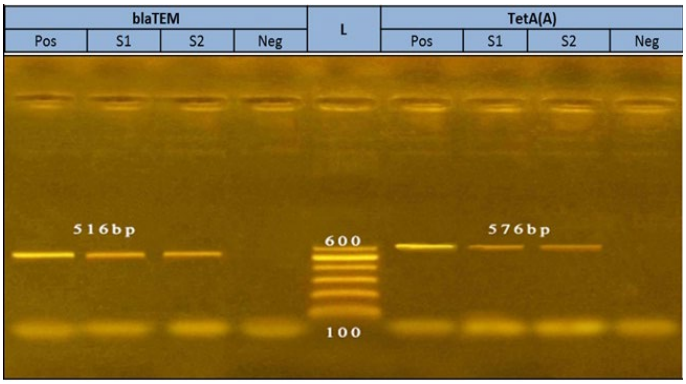


Fig. 5. Electrophoresis of detected antimicrobial resistance gene deoxyribonucleic acid fragments of *P. aeruginosa* amplified by (PCR) from strains (*tetA(A)* – 576 bp) and (*BlaTEM*- 516bp).

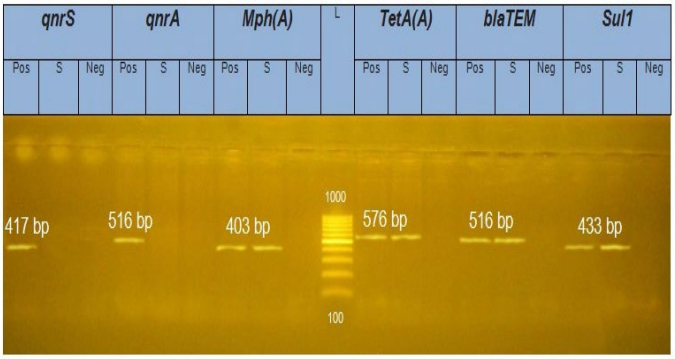


Fig. 6. Electrophoresis of detected antimicrobial resistance gene deoxyribonucleic acid fragments of *Enterobacter* speceis amplified by (PCR) from strains (*tetA(A)* – 576 bp), (*BlaTEM*- 516bp), (*Mph(A)* – 576bp), (*QnrA* – 516 bp), (*QnrS*- 417bp), and (*Sul1* – 433bp).

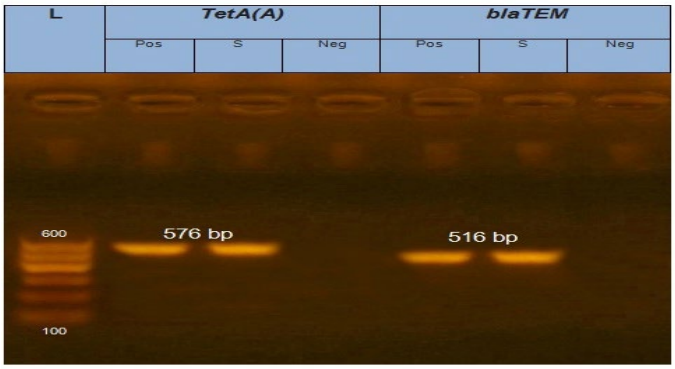


Fig. 7. Electrophoresis of detected antimicrobial resistance gene deoxyribonucleic acid fragments of *R. ornithinolytica* amplified by (PCR) from strains (*tetA(A)* –576 bp) and (*BlaTEM*- 516bp).

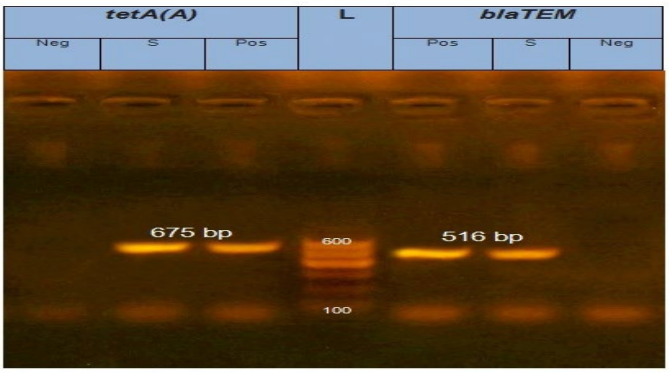


Fig. 8. Electrophoresis of detected antimicrobial resistance gene deoxyribonucleic acid fragments of *S. paucimobilis* amplified by (PCR) from strains (*tetA(A)* – 576 bp) and (*BlaTEM*- 516bp).

## DISCUSSION

Subclinical mastitis is a hygienic and legal problem for dairy producers and causes financial loss. It is described by elevated somatic cells, particularly the mammary tissue leukocytes, which provide an allusion to the gland's inflammatory reaction. The negative impact on milk quality is the principal sequel of bovine udder inflammation and involves a pathogens repository that disseminates infection to surrounding animals (Jagielski *et al*., 2019; Youssif *et al*., 2020).

In the investigated farm, 444 out of 695 examined lactating cows were positive for subclinical mastitis (63.88%) at the cow's level. This high prevalence covers one year of examination. The outcome was consent (Suleiman *et al*., 2018). The comparatively lower results were reported by Krieger *et al*. (2017), while the elevated prevalence was recorded by Langer *et al*. (2014). Coliforms cause up to twenty-five percent of bovine mastitis and proliferate in contaminated water and droppings. The elevated percentage of SCM due to Coliforms signalizes poor sanitary production. They invade the cow's mammary glands by the sphincter of the teat when the end of the teat touches these microorganisms through the environment (Ssajjakambwe *et al*., 2017). *Klebsiella* mastitis has a problem in farms, which use sand coverings; this is due to the fecal release of this bacterium by non-diseased animals. Oral intake of *Klebsiella* could be due to organism presence in crops or contamination of water by feces (Masiello *et al*., 2016).

*P. aeruginosa* SCM has elevated in places, causing major damage and this type of SCM is traced to polluted water sources. Parlor floor contamination with foremilk also accelerated its transmission. Edible items, sewage, and water are significant sources of *C. amalonaticus*. It releases intestinal toxins working on the mucosa of the intestine. *E. cloacae* are considered the major genus species and are commonly isolated from mastitis samples of milk (Jean *et al*., 2002; Youssif *et al*., 2021b).

*Raoultella* was categorized previously as part of the genus *Klebsiella*. With more detailed examination, several isolates identified earlier as *Klebsiella* spp. may be reported, as *Raoultella* spp. (Masiello *et al*., 2016). *Sphingomonas* spp. is inserted from the surroundings. It has a high pathogenicity and causes severe clinical expression. *S. paucimobilis* possesses at least two different kinds of sphingolipids. It was detected in milk with elevated somatic cell count (Cengiz *et al*., 2015).

Non-aureus *Staphylococci* were associated with cows' mastitis with obvious udder signs (Riekerink *et al*., 2008; Saini *et al*., 2012). They propose reasonable universal dissimilarities among countries, grazing lands, and between investigations at different times on the same farm regarding the bacteria causing clinical and subclinical mastitis. Also, Gram-negative bacteria associated with SCM in the study may be attributed to a recent infection with these species.

The expansion of antimicrobial resistant (AMR) bacteria and their genes to people and animals occurred in many manners of transmission, such as the utilization of insufficiently cooked food, handling of raw edible material, or through the environment as contaminated water and soil. This usual spread can influence the environment and adjacent communities of people. AMR can happen through deoxyribonucleic acid mutations or via antibiotic alteration by modifying enzymes (Younis *et al*., 2018). Biofilm existence in the mammary glands results in a lower effectiveness of antimicrobial therapy leading to continual infections, which are produced by the sub-inhibitory concentrations of various antimicrobials and have a role in transmitting resistance between microbes (Tawheed *et al*., 2018).

The results were related to the resistance reaction of *S. chromogenes* isolates toward selective antibiotics following the results mentioned by Abera *et al*. (2013); Kateete *et al*. (2013), while the results in Table 2 agreed with those reported previously by Mia *et al*. (2017). The examined twenty isolates of *Klebsiella oxytoca* showed 100.0% sensitivity toward amikacin, norfloxacin, and enrofloxacin. However, fifty % of *Klebsiella oxytoca* isolates were sensitive to cefoxitin and gentamicin (Table 3), this result is in line

with Kateete *et al*. (2013).

*Pseudomonas aeruginosa* isolates were 100.0% sensitive to gentamicin, nitrofurantoin, amikacin, enrofloxacin and norfloxacin. The data presented in Table 3 concluded that nitrofurantoin, trimethoprim/ sulfamethoxazole, enrofloxacin, and norfloxacin were the most effective antibiotics against *Sphingomonas paucimobilis*, followed by cefoxitin, gentamicin, and amoxacillin+ clavulanic acid in a percentage of 50.0. On the other hand, gentamicin was the most effective antibiotic against *Enterobacter* species followed by clindamycin, amikacin, and enrofloxacin. This result was nearly comparable to the result of Chamlagain (2011) and Cengiz *et al*. (2015).

For *Raoultella ornithinolytica* isolates, cefoxitin, gentamicin, amikacin, enrofloxacin, and amoxacillin+ clavulanic acid were the most effective antibiotics. These results were nearly comparable to the results of Kaya *et al*. (2015) and Abd Ali and Al Ali (2017). Also, gentamicin and norfloxacin were the most effective antibiotics for *Citrobacter amalanaticus* isolates (Table 3). These results are comparable to the results of Abera *et al*. (2013). On the other hand, the two examined species were 100.0 % resistant to tetracycline and penicillin antibiotics. These results are compatible with the finding recorded by Kateete *et al*. (2013) and Hleba *et al*. (2014).

Commonly, antimicrobials applied for curing udder infection are β-lactams. These clarify that bad utilization of antimicrobials is an agent that can cause elevated pathogens resistance. Cytoplasmic exocytosis channels and deactivation of enzymes are means for bacterial resistance to tetracycline. The TetA gene is associated with resistance to tetracycline and takes part in antimicrobials elimination from cells (Arab *et al*., 2018).

The results of the examined *Staphylococcus chromogenes*, *Klebsiella oxytoca*, Bacillus species, *Pseudomonas aeruginosa*, *Sphingomonas paucimobilis*, *Enterobacter* species, *Raoultella ornithinolytica*, and *Citrobacter amalanaticus* isolates showed high resistance to beta-lactams and tetracyclines. They expressed this by encoding the resistance genes (*blaZ*, *BlaTEM*, and *bla* for B-lactams) and (tetK and *tetA(A)* for tetracyclines), Table 4. Das *et al*. (2017) concluded that among Gram-negative (Pseudomonas, *Klebsiella*, and *Enterobacter*), which were isolated from SCM bovine milk samples, 48.0% (24/50) were found tetracycline-resistant and 6 (12.0%) harbored *BlaTEM* genes in PCR. Furthermore, five (10.0%) isolates carried the tet(A) gene.

*Klebsiella oxytoca*, *Enterobacter* species, and *Citrobacter amalanaticus* isolates were positive for the *Sul1* gene in a percentage of 50, 100, and 100, respectively, which is responsible for resistance against sulfa-related antibiotics, these results are in line with Chamlagain (2011) and Priya and Ayodhya (2016).

Coagulase Negative *Staphylococci* have become widespread pathogens recovered from bovine mastitis, and subclinical forms are even described as emerging pathogens. The *BlaZ* gene is responsible for staphylococcal resistance to β-lactams. Moreover, the significant participation of β-lactam in udder inflammation control becomes a threat factor to mastitis therapy effectiveness and community health by *blaZ* transferability among *Staphylococci* of both people and animals (Sawant *et al*., 2009; Waller *et al*., 2011). While 58.9 % of *Staphylococci* isolates were positive for penicillinase production (Abrahmsén *et al*., 2014). Björk *et al*. (2014) found that Coagulase Negative *Staphylococci* are the most microbes causing SCM in bovine in a percentage of 31.7, eighty percent of them were positive for β-lactamase (β+). While Srednik *et al*. (2017) revealed that 90 isolates of CNS were isolated from IMI bovine in Argentina, *blaZ*, *MecA*, and *MecC* genes were detected in 21, 4, and 1 isolate of CNS, respectively.

The *MecA* gene in Methicillin-Resistant Coagulase Negative *Staphylococci* (MRCNS) acts resistant to methicillin/oxacillin. The bacterial protein synthesis silence is the action mode of macrolides (Stevens *et al*., 2018). *E. cloacae* were positive for the *Mph(A)* gene, which is responsible for resistance against erythromycin. The resistance of the isolated bacteria against one or more antibiotics signifies that the microbes may have genetically or gained more than one resistance gene, which is responsible for the lesser effect against them, and this is explained by extensive misuse of these drugs (Eputiene *et al*., 2012).

Misuse of antimicrobials for the control of diseases of dairy bovine could have harmful effects on animal and human health. Antimicrobial resistance is one of the hazards of the 21st century. The resistance effect is characterized by elevated hospital stay length, price, and mortality due to inappropriate initial antimicrobial treatment. Antimicrobial resistance refers to lower sensitivity or total insensitivity of some pathogens to one or more antimicrobial agents (Jayarao *et al*., 2019). Resistance to antibiotics happens due to drug deactivation, reduced accumulation owing to decreased permeability, elevated efflux, mutation outcome, or obtaining external resistance genes (Bengtsson-Palme, 2018).

Thus, the movable resistance genes that are circulating among human microbes may re-emerge during therapy. The induction of new further resistance genes into pathogens causes destructive outcomes for people's health. Antimicrobial resistance takes place when bacteria acclimate to antibiotics, leading to drug incompetence and continual infections. The application of the HACCP system is necessary for achieving safe milk production (Gamal *et al*., 2022; Ibrahim *et al*., 2022).

## CONCLUSION

From the study results, it is concluded that the widespread AMR less common Gram-negative species causes SCM and their genes in the examined cow's milk. This high spread may lead to transferring of AMR from the animal-to-human community, which is represented as, a significant public health concern. The proper dose and duration of antibiotics intra-mammary infusion should be taken under veterinary supervision.

## CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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