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DETERMINANTS of INFECTION as GENETIC INDICATORS in MASTITIS in COWS

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RESEARCH ARTICLE

Abstract

The resistance to antimicrobial substances severely impacts public health and the abuse of antibiotics leads to antimicrobial resistance (AMR) or the antibiotic "resistome" (Wright, 2007). Bovine mastitis is largely diagnosed in dairy farms and is caused by a variety of pathogens including *Streptococcus spp*., *Staphylococcus spp.* and *Escherichia coli*. AMR gene expression testing in bacteria involved in mastitis in dairy cows was performed. Milk samples were subjected to the California Mastitis Test. Positive samples were transferred using eSwab, cultured on Columbia blood agar and after isolation, also on MacConkey agar. The Qiagen DNeasy kit was used for DNA extraction and qPCRs were run using an Agilent thermocycler. In most of the samples tested (n = 42, from three different lactating farms), the presence of *ampC* (36 out of 42; 85.7%) and *blaZ* (95.2%),correlated with confirmed resistance to beta-lactam and cephalosporin antibiotics. A variable presence of other tested AMR genes was detected, including *ermB*, resistance to lincosamide and macrolide (35.7%), *ermC* (28.6%), erythromycin resistance, methicillin-resistant *mecA* (42.9%), and tetracycline-resistant *tetK* (78.6%). The phenomenon of antimicrobial resistance is present in dairy farms in West Romania. A large number of AMR genes were detected in the samples tested, with the highest resistance observed to beta-lactam antibiotics and cephalosporins.

Keywords:antimicrobial resistance;gene expression; mastitis

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INTRODUCTION

Antibiotic resistance is currently one of the biggest challenges in medical and veterinary profession. It has a huge impact on choosing the appropriate approach towards the treatment of primary bacterial infections and also for secondary bacterial infections developing as a consequence of viral and fungal infections. This resistance phenomenon has been reported not only against natural, semi-synthetic and completely synthetic antibiotics, including those which do not enter eukaryotic cells. The rapid spread of antibiotic resistance is facilitated by horizontal gene transfer. The pace of discovery of new antibiotic compounds does not keep pace with emerging antibiotic resistance. The current understanding of antibiotics is changing to possible sources of nutrition for the bacteria or they are viewed as inter-microbial signalling agents, rather than weapons against fighting bacterial infections.

From their introduction as a therapeutic/prophylactic tool, antibiotics have been successful in reducing mortality, but not the persistence of infectious diseases. The excessive use and misuse of antibiotics are the main causes of the development of resistance and it is considered an evolutionary mechanism in which bacteria adapt to exposure to new environments and molecules. Chromosomal mutations and genes imported through genetic recombination are also important in the spread of antimicrobial resistance (EL-Halfawy and Valvano, 2012; Tavares et al., 2013). The phenomenon of antimicrobial resistance has huge significant for public and animal health.

It has been shown previously that bacteria may acquire resistance in natural environmentssuch as soil (Josephson, 2006; Wright, 2007). The framework for understanding the ecology of resistance on a global scale can be described by the concept of the antibiotic resistome. The resistome can be defined as the collection of all antibiotic resistance genes including those circulating in pathogenic bacteria, antibiotic producers and benign non-pathogenic organisms found either free living in the environment or as commensals of other organisms (D'Costa, 2006). These so-called antibiotic producers live in soils and are responsible for killing most of the bacteria living in their vicinity. However, some of the bacteria start developing resistance to these natural products (Wright, 2007).

Several different strategies are known to be employed by bacteria for induction of antibiotic resistance. It may be acquired by spontaneous mutation in the coding gene of the target protein. The result of this action is the reduction or lack of affinity to the antibiotic. Also, horizontal transfer of antibiotic resistance genes from other bacteria occurs (Hassan et al., 2012).

The mechanisms in which an antibiotic-resistance gene product may take action include enzymatic degradation of the antibiotic, altering the antibiotic target site or pumping the incoming antibiotic out of the cell by a transport mechanism. All these processes contribute to the production of infection that is very difficult to treat as it produces highly resistant bacteria such as *Escherichia coli* or methicillin-resistant *Staphylococcus aureus* (MRSA) (Overbye and Barrett, 2005; Reynolds et al., 2004).

The mechanism of inactivation of antibiotics by bacteria containing specialised enzymes is well documented and the best example is β-lactamase. This enzyme cleaves the β-lactam rings and therefore inactivates β-lactam antibiotics. Development of resistance to fluoroquinolones, aminoglycosides and penicillin results in the reduced ability to enter the cell, but it may be overcome by increasing the drug concentration. Bacteria containing genes resistant to tetracyclines increase removal of this antibiotic. Also, erythromycin, chloramphenicol and ciprofloxacin have developed similar mechanisms of resistance. The activity of some antibiotics can be blocked by structural changes in bacteria, e.g. proteins responsible for cell wall synthesis of the enterococci have low affinity for cephalosporins. Resistance genes carried on plasmids can also be implicated in the elimination of the binding site which results in resistance to macrolide and lincosamide. Additionally, alternative binding sites can be produced making bacteria resistant to the action of antibiotics (Levy, 2002).

Antibiotic resistance is appreciated as an existential threat to mankind (Report, 2014a). This report indicates that reduced use and greater legislative control will reduce the increase in resistance but it will not tackle the problem of resistance which already exists. International institutions such as WHO advocate the exploration of new approaches to reducing resistance (Report, 2014b). Interest in the use of bacteriophages for controlling bacterial infections has been again increasing for the last 20 years (Barrow & Soothill, 1997; Abedon, 2017) and phages for which the sex pili of transmissible antibiotic resistance plasmids are the receptors may be a positive way forward in selecting for loss of AMR plasmids and driving microbial evolution back towards antibiotic sensitivity (Colom et al., 2020).

Bovine mastitis constitutes one of the most economically important bacterial diseases in dairy farming. The disease itself involves the inflammation of the mammary glands induced most frequently by a bacterial intramammary infection caused by various bacterial strains including *Streptococcus*, *Staphylococcus* and *Escherichia*. The occurrence of uncontrolled mastitis infections increases the treatment cost and causes losses in milk production due to increases somatic cell count and bacterial total plate count. The use of antibiotics in the treatment of bovine mastitis is implicated in the occurrence of resistant bacteria within the food chain and may lead to drug residues in milk. Milk is then consumed by humans with potential adverse effects on their health. Hypersensitivity reactions may be produced in susceptible humans by the presence of drug residues and antibiotic resistance may also be selected. Another important of aspect of the presence of drug residues involves altering the quality of raw milk especially when using starter cultures for the production of cheese and yoghurt (Oliver et al., 2012).

Ultra-high pasteurisation treatment is important in the process of destroying pathological bacteria; however, it does not reduce or remove the residual drugs. For the protection of humans against harmful effects of antibiotic residues in milk, Food and Agriculture Organization (FAO) and European Union (EU) have set maximum residue levels (MRLs) of 1.5, 0.2 and 4.0 μg/mL for the milk residues of neomycin, streptomycin and penicillin G (Babapour et al., 2012; Park et al.,2016).

Some of the most popular antibiotics used in the treatment of bovine mastitis include:

i) penicillin G, which supresses Gram-positive bacterial proliferation by interfering with cell wall assembly,

ii) streptomycin, which inhibits bacterial growth by interfering with peptide synthesis systems of bacteria; and

iii) neomycin, which acts by binding the region for translation of mRNA and message readout and disrupting its functions (Park et al., 2016).

Therefore, it is very important to control how antibiotics are utilised in the treatment of bovine mastitis, to protect both animals and humans. Moreover, it may often happen that the random choice of antibiotics used for treatment without proper testing may not reduce the severity of infection and will only increase the amount of drug residues in milk.

From the point of view of a farmer, the production and delivery of maximum quantities of high-quality milk is a very important objective. Bovine mastitis is the most critical factor resulting in the loss of milk quality. It has been previously demonstrated that udders are more susceptible to new intramammary infection during the early dry period (when there is no lactation). Another critical point with increased susceptibility to mastitis is in the period near calving. This is thought to relate to physiological changes occurring in the mammary gland, either from or leading up to milk production. Therefore, the early and the late part of the dry period are considered the most important times for the control of bovine mastitis. It is advised that all mammary quarters of all cows maintained together should be treated with antibiotics approved for use in dry cows after the last milking of lactation. This process is applied in order to eradicate infections present during late lactation and for the prevention of new infections during the early dry period, a period with the highest susceptibility to new infections. The control of mastitis during the dry period is also achieved through antibiotic therapy at drying off (Ruegg, 2013).

Antibiotics which are the most commonly used in dairy farming include penicillin, cephalosporin, streptomycin and tetracycline. The administration of antibiotics to entire herds is often practised as a prevention strategy. General advantages of using antibiotics in dairy farming involve healthier and more productive cows, decreased incidence of disease, reduced mortality, decreased exposure to pathogens and the production of large quantities of high-quality milk destined for human consumption. Although, there are multiple benefits of using antibiotics in dairy farming this approach is also, to a greater extent, responsible for the emergence of antimicrobial resistance (Oliver et al., 2012).

The present study aimed to highlight the expression of genes encoding antimicrobial resistance in some herds of dairy cows in Romania and to indicate the genes that generated the most intense resistance to substances with antimicrobial action (screening for AMR of bacteria involved in dairy cows mastitis by gene expression testing: *ampC* - resistance to beta-lactam antibiotics and cephalosporins, *blaZ* - resistance to beta-lactam antibiotics, *ermB* - resistance to lincosamide, macrolide, *ermC* - erythromycin resistance, *mecA* - resistance to methicillin, and *tetK* - resistance to tetracycline).

MATERIALS AND METHODS

Bacterial sample collection

Samples were collected from the bovine teat canal with eSwab and were cultured on blood culture medium – Columbia blood agar and incubated at 37ºC for 24 hours. After the incubation period, the bacterial colonies were isolated, Gram-positive bacteria were replicated on blood agar media (5% ram blood) -Columbia blood agar, and Gram-negative bacteria on MacConkey agar (Hutu et al., 2019). Plates were incubated for 24 hours at 37ºC. Bacterial colonies werepicked into Eppendorf tubes with 0.5ml of culture broth.

DNA extraction

DNA was extracted using QIAGEN DNeasy Blood and tissue extraction kit as per manufacturer's instructions. DNA concentration was quantified using a NanoQuant plate (Tecan) and the absorbance at 260nm/280nm was measured to indicate the purity of DNA (1.6-1.8 indicates relatively pure DNA). DNA samples were stored at 20ºC.

qPCR reaction set up

Quantitative PCR reactions were performed using an Agilent thermocycler. For each 20 µl of the total reaction, 12.5 µl of SYBR Green mix (Agilent), 1 µl of each, forward (FW) and reverse (RV) primers (Table 1.), 25ng of bacterial DNA and water, were mixed. Master mix constituting of SYBR Green, FW and RV primers was made for each primer set. Water was adjusted accordingly depending on the concentration of DNA of given sample. Genes tested in the project included:

- *ampC* – resistance to beta lactam antibiotics, cephalosporins,
- *blaZ* – resistance to beta lactam antibiotics,
- *ermB* – resistance to lincosamide, macrolide,
- *ermC* – resistance to erythromycin,
- *mecA* – resistance to methicillin,
- *tetK* – resistance to tetracycline.

Name	Sequence (5' to 3')
ampC_FW	TGAGTTAGGTTCGGTCAGCA
Amp C RV	AGTATTTTGTTGCGGGATCG
blaZ FW	ACTTCAACACCTGCTGCTTTC
blaZ RV	TGACCACTTTTATCAGCAACC
aac(6')aph(2") FW	GAAGTACGCAGAAGAGA
aac(6')aph(2") RV	ACATGGCAAGCTCTAGGA
tetK FW	GTAGCGACAATAGGTAATAGT
tetK RV	GTAGTGACAATAAACCTCCTA
ermC FW	ATCTTTGAAATCGGCTCAGG
ermC RV	CAAACCCGTATTCCACGATT
ermB FW	CATTTAACGACGAAACTGGC
ermB RV	GGAACATCTGTGGTATGGCG
mecA FW	CTGATGGTATGCAACAAGTCG
mecA RV	TGAGTTCTGCAGTACCGGATT

Table 1. Antimicrobial resistance (AMR) gene primer sets used.

RESULTS AND DISCUSSIONS

The initial steps of the project involved setting up protocols for the molecular biology techniques which were going to be utilised. The most crucial steps included a successful bacterial DNA extraction from a relatively small sample volume, setting up the equipment for the assessment of DNA concentration and quality, obtaining a sufficient DNA yield and DNA of acceptable quality, testing antimicrobial resistance gene primer sets, testing the qPCR protocol.

After performing the bacterial DNA extraction, to verify the yield and the quality of samples analysed in the project, Tecan NanoQuant spectrophotometer was tested using DNA samples of known concentrations, previously measured by NanoDrop used in another laboratory. The calibration of the machine was confirmed by similar readouts of DNA concentration and DNA quality.

Testing of the qPCR amplification was performed using *Staphylococcus aureus* standard DNA sample (supplied by ATCC) with aminoglycoside resistance gene primers (*aac(6')aph (2")).* It has been shown previously that *S. aureus* is resistant to aminoglycoside medication, hence this choice of technique for the validation of the protocol (Schmitz et al., 1999).

After performing the qPCR reaction, the DNA amplification curve starting at the 12th cycle during the annealing step of PCR reaction confirmed the accuracy and correctness of the experimental set-up (Figure 1.).

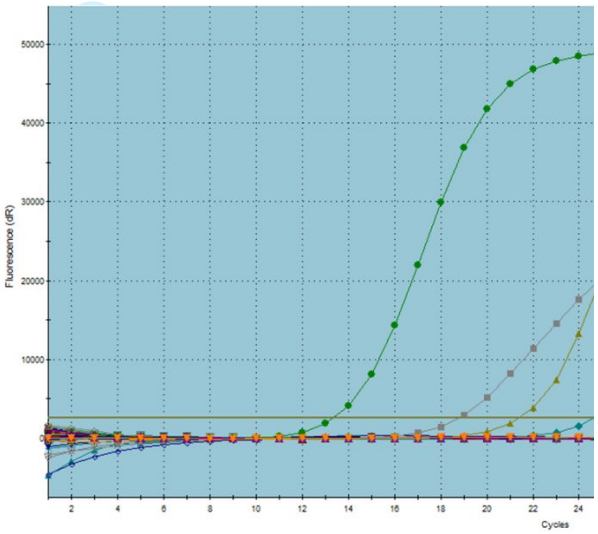
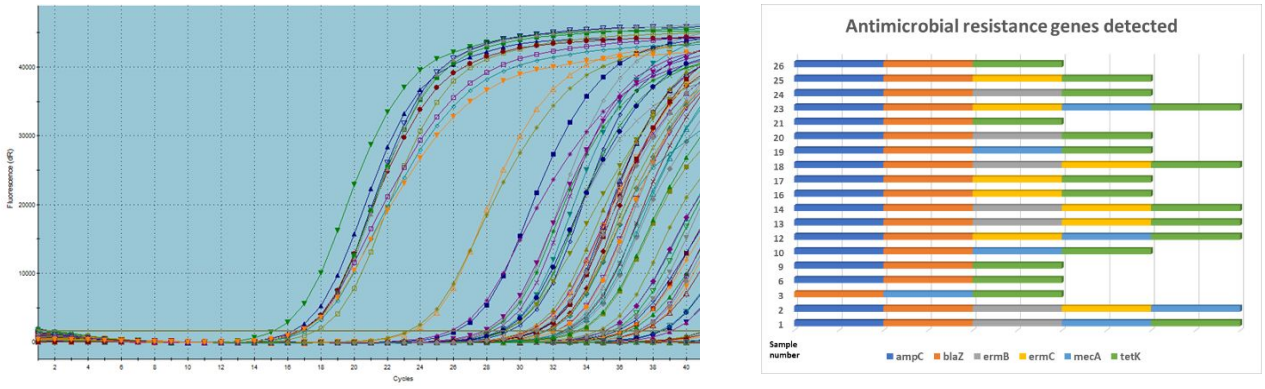


Figure 1. Testing of qPCR protocol using *Staphylococus aureus* DNA (standard bacterial DNA supplied by ATCC company) using *aac aac(6')aph (2")* primer set. There was a predicted amplification of aminoglycoside resistance gene previously shown in published literature (Schmitz et al., 1999).

After initial testing of the protocol, the bacterial DNA was extracted from the sample cultures taken from the teat canal and the DNA concentration obtained was satisfactory for the purpose of setting quantitative polymerase chain reaction. Any samples with amplification starting at later than 40th cycle of the annealing step of PCR reaction were considered negative (Figure 2a.).There were multiple resistance genes detected in each sample tested (Figure 2b.)



(a) (b)

Figure 2. (a) Representative amplification plot of samples tested for the presence of following AMR genes (*ampC* – beta lactam antibiotics, cephalosporins, *blaZ* – beta lactam antibiotics, *ermB* – lincosamide, macrolide, *ermC* – erythromycin, *mecA* – resistance to methicillin, *tetK* – resistance to tetracycline). (b) Representative image of AMR genes detected in mastitis milk cultures.

In most of the samples tested (n=42, from three different dairy farms), resistance genes *ampC* (36 out of 42; 85.7%) and *blaZ* (40 out of 42; 95.2%) were present signifying resistance to treatment with beta lactam antibiotics and cephalosporins. There was a variable presence of other AMR genes tested including *ermB*, which confirms resistance to lincosamide and macrolide (15 out of 42; 35.7%), *ermC*, with erythromycin resistance (12 out of 42; 28.6%), *mecA*, with methicillin resistance (18 out of 42; 42.9%) and tetracycline resistant - *tetK* (33 out of 42; 78.6%) (Figure 3.).

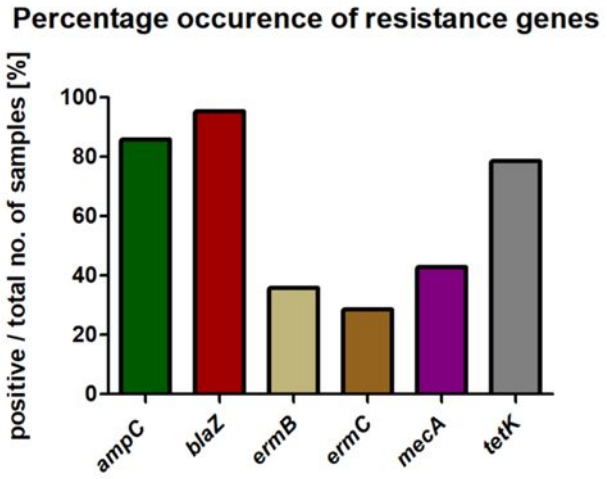


Figure 3. A visual representation of the percentage of samples positive for AMR genes versus the total number of samples.

In tested samples, a large number of antimicrobial resistance genes was found. Resistance to beta-lactam and cephalosporin antibiotics was most observed. Moreover, tetracycline, methicillin, lincosamide, macrolide and erythromycin resistance genes were also reported.

This study confirms that the phenomenon of antimicrobial resistance is prevalent in dairy farms in Romania and discourages the use of antibiotics in mastitis management. It also highlights the usefulness of AMR genetical

analysis in providing data which can be used to formulate indications and guidance to farmers in choosing the best mastitis treatment and prevention options.

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Conflicts of Interest
The authors declare that there is no conflict of interest.

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