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THE INFLUENCE OF TREATMENT AND HEALTH CONDITION OF THE ANIMAL ON DRUG EXCRETION IN BOVINE MILK

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Doctoral dissertation

VPLIVI ZDRAVLJENJA IN ZDRAVSTVENEGA STANJA ŽIVALI NA IZLOČANJE ZDRAVIL Z MLEKOM PRI KRAVAH

Doktorska disertacija

Ljubljana, 2020

Aleksandar Siljanoski

The influence of treatment and health condition of the animal on drug excretion in bovine milk

The dissertation has been carried out at the Institute of food safety, feed and environment, at the Veterinary Faculty, University of Ljubljana.

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I hereby declare that the doctoral dissertation is exclusively the result of my own original research, the results are accurately mentioned and no any third person's copyright is violated.

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ABSTRACT

Key words: Intramammary drug, intramammary preparation, combined antimicrobial therapy, veterinary drug residue, dairy cow, screening test.

The purpose of this study was to determine antimicrobial residues in milk from dairy cows treated for clinical mastitis. Milk samples were collected from udder quarters from 97 cows treated with intramammary drugs and simultaneously with other drugs via the systemic route. Data on cow health and used drugs were obtained from the farm veterinarians. Milk samples from 66 affected udder quarters were taken for bacterial identification before treatment. In many of the treated cows withhold period (WP) of 14 milkings was prescribed due to the Slovenian National Legislation for off-label use of drugs. The milk samples from treated udder quarters, taken before and after the prescribed WP, were analysed for antimicrobial residues. Additionally, milk samples from 48 cows were taken from untreated healthy udder quarters to check if any crossover of drugs had occurred. Three screening tests were used for antimicrobial detection. The positive samples were analysed with the appropriate confirmatory method. In 22 (22.7%) cows the milk samples from the treated infected quarters contained antimicrobial residues above the maximum residue limit (MRL) after the WP of 14^{th} milking. Thirteen milk samples had tetracycline residues above the MRL, 6 amoxicillin, 4 cefquinome and 1 neomycin. Antimicrobial residues were not detected in milk samples from the untreated quarters. Out of 12 cows treated intramammarily with dihydrostreptomycin instead of systemic as it is intended, 7 (58.3%) milk samples had residues above the MRL after the 14^{th} milking. The treatment interval differed significantly ($p \leq 0.05$) between the positive and the negative milk samples treated with tetracycline, cefquinome and amoxicillin. In our study cows treated at 12 hour interval had higher chances of having residues. The mastitis caused by *E. coli* was statistically significant ($p \leq 0.05\%$) between the positive milk samples and the negative milk samples. No significant differences were found between the positive and negative cows regarding the combination antimicrobial therapy.

IZVLEČEK

Ključne besede: intramamarna zdravila, intramamarni pripravki, kombinirano antimikrobno zdravljenje, zaostanki veterinarskih zdravil, presejalni testi, krave molznice.

V raziskavi smo določali prisotnost antimikrobnih ostankov v mleku pri kravah molznicah, ki so bile zdravljene zaradi kliničnega mastitisa. Vzorci mleka so bili zbrani iz obolelih vimenskih četrti 97. krav, ki so bile zdravljene istočasno z intramamarnimi zdravili in zdravili za paranteralno uporabo. Podatke o zdravstvenem stanju krav in uporabljenih zdravilih so nam posredovali lečeči veterinarji. Za identifikacijo povzročiteljev infekcij je bilo odvzetih 66 vzorcev mleka iz okuženih vimenskih četrti pred zdravljenjem . V večini primerov je bila predpisana karenca 14 molž, zaradi izjemne uporabe zdravil. Vzorce mleka, ki so bili odvzeti iz zdravljenih vimenskih četrti pred in po predpisani karenci, smo preiskali na antimikrobne zaostanke. Pregledali smo tudi vzorce mleka, odvzete iz zdravih vimenskih četrti pri 48. zdravljenih kravah, zaradi preverjanja morebitne možnosti prisotnosti zdravil. Vzorce mleka smo najprej analizirali s tremi različnimi presejalnimi testi, pozitivne vzorce smo ponovno analizirali z ustrezno potrditveno metodo. V 22. primerih (22,7%) smo v mleku iz zdravljenih četrti ugotovili prisotna zdravila nad MRL-vrednostjo po 14. molžah. V 13. primerih so to bili tetraciklini, v 4 primerih cefkvinom, v 6 primerih amoksiciklin, ter v 1 primeru neomycin. Ostankov antimikrobnih zdravil ni bilo v vzorcih iz nezdravljenih vimenskih četrti. Interval dajanja zdravil se je značilno razlikoval ($p \leq 0.05$) med pozitivnimi in negativnimi vzorci mleka pri kravah zdravljenih s tetraciklinom, cefkinomom in amoksiciliom. Pri kravah, ki so bile zdravljene dvakrat na dan, obstaja večja verjetnost, da bodo ostanki zdravil presegali MRL-vrednost po sedmem dnevu končanega zdravljenja. Pri okuženih kravah z *Escherichia coli* obstaja statistično večja verjetnost ($p \leq 0.05$) daljšega izločanja antimikrobnih zdravil.. Glede na kombinirano antimikrobno terapijo med pozitivnimi in negativnimi kravami ni bilo statistično značilnih razlik.

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ABBREVIATIONS

A. schindleri	*Acinetobacter schindleri*
ADI	Acceptable Daily Intake
Amo	Amoxicillin
CNS	Coagulase-negative staphylococci
Dhs	Dihydrostreptomycin
E. coli	*Escherichia coli*
EU	European Union
Gen	Gentamicin
IMI	Intramammary infection
Imm	Intramammary
K. pneumoniae	*Klebsiella pneumoniae*
LPS	Lipopolysaccharide
MRL	Maximum Residue Limit
na	not analysed
ns	not sampled
NSAID	Nonsteroidal anti-inflammatory drug
OTC	Oxytetracycline
PABA	Para-aminobenzoic acid
Pb	Procaine benzylpenicillin
PLC	Plate Loop Count
S. aureus	*Staphylococcus aureus*
SCC	Somatic Cell Count
SCP	Summary Product Characteristics
SF	Safety factor
Str. uberis	*Streptococcus uberis*

TC	Tetracycline
TTSC	Time to Safe Concentration
WP	Withhold Period

1. INTRODUCTION

Milk, together with dairy products, constitutes a large portion of the average daily intake per capita in Europe (more than 150 kg milk per capita/year). It is also an indispensable nutrient for normal development throughout life especially in younger age (IFCN, 2006; Weaver et al., 2013). Animals used for milk production often suffer from mastitis, inflammation of the mammary gland. Major consequences of mastitis in dairy cattle are lower milk yield and shortened productive lifespan of the affected cows causing significant economic losses (Rajala-Schultz et al., 1999; Petrovski et al., 2006; Halasa et al., 2007). In mastitis treatment, intramammary infusion is the most common route of applying a drug. This delivers a high drug concentration directly into the infected udder. However, such a route of administration often results in presence of drug residues in milk (Kang et al., 2005). Each drug has a prescribed period of withhold of milk from sale to avoid antimicrobials in the milk supply. Violative residues following intramammary treatment of mastitis may arise following inappropriate treatments records, inability to identify and withhold milk from treated cows, too short withhold period (WP), off-label application and combination usage (Booth, 1982; Oliver et al., 1990; McEwen et al., 1992; FDA, 2015). Antimicrobial residues in milk are of concern because of the risk of developing resistant microorganisms, allergic reactions in milk consumers, and inhibition of starter cultures used in the dairy food industry (Allison, 1985; Seymour et al., 1988; McEwen et al., 1991).

Some studies have reported prolonged excretion of antimicrobials in milk after treating cows for mastitis. However, to our best knowledge, reports describing excretion of antimicrobial residues in milk from individual quarters in cows treated off-label as well as with combined antimicrobial therapy do not exist. The pharmacokinetic properties of a drug applied through either the intramammary, or systemic route is greatly affected by the pH of the medium and the dissociation constant (pKa) of the drug, as well as the ability of protein binding (Ziv, 1975). Differences occur in the physico-chemical characteristics between the healthy milk and milk from mastitic udder, with most of the pharmacokinetic studies being performed in healthy cows. Studies performed in mastitic cows showed slower drug elimination. Poelarends et al. (2001) noted no significant

differences of WP after threating cows for mastitis with combination therapy or single intramammary therapy. However, the mean excretion time was longer for combination therapy. Slower elimination of azithromycin from milk of mastitic quarters was noted after treating bovine subclinical mastitis *via* the intramuscular route (Lucas et al., 2009). Different pharmacokinetic properties of norfloxacin were noted after intramuscular treatment of lactating cows suffering from clinical and subclinical mastitis (Gips and Soback, 1999).

Milking frequency, dose infused and udder health status were associated with the crossover of erythromycin into the healthy untreated quarters after intramammary administration of the drug in lactating dairy cows with specific mastitis. However, no residues were found beyond the first milking (Bansal et al., 2010).

Change in elimination kinetics are not restricted to antimicrobials only. Elimination kinetics of other drugs are also affected. For example, prolonged clearance from the body of mastitic cows have been reported after systemic use of non-steroidal anti-inflammatories (Lohuis et al., 1991; Kissell et al., 2015).

These studies suggest that antimicrobial drugs in therapy of mastitis in dairy cows pose risk of antimicrobial residues in milk after the prescribed WP. Treatment of mastitis with a combination of antimicrobial drugs is frequently practiced in Slovenia and elsewhere (du Preez, 1988; Oliveira and Ruegg, 2014).

In order to protect public health and prevent any adverse effects Maximum Residues Limits (MRL) for pharmacologically active substances are laid down in the European Union (EU) by Commission Regulation (EU) 37/2010 (European Commission, 2010). The WP, indicated in the instructions' label for a medicine should be long enough for any residues in animal products intended for human consumption to fall below the MRL. Investigations concerning the WP are encouraged by Directive 2001/82/EC (EC, 2001), but persistence of antimicrobial residues in milk after the WP in dairy cows is a problem reported for decades (Johnson et al., 1977; Oliver et al., 1984; Seymour et al., 1988; McEwen et al., 1992; Podpečan et al., 2014).

Different methods are available and used to establish WPs in milk (EMEA, 2000; Vranic et al., 2002). According to the European Medicinal Agency the harmonized method in the EU for determining WPs is the "Time to Safe Concentration" (TTSC). It determines the time necessary

for the measured concentration to drop below the MRL, and stay below the MRL at later times. The residues are determined individually; healthy animals are given the product containing the drug in question and are milked twice a day (EMEA, 2000). The WP is evaluated by the manufacturer of the drug preparation.

Bulk tank milk occasionally tests positive to antimicrobials, but far less frequently than in the past. Slow and prolonged excretion of antimicrobials in milk after mastitis treatment has been reported in a few published studies but has not received much attention (McEwen et al., 1991, 1992; FDA, 2015). If the excretion of antimicrobials is slower in treated mastitic quarters, bulk tank milk may be contaminated with antimicrobial residues even when the prescribed WP has been respected.

Before the milk is placed on the market or sent for further processing, producers in the dairy industry must be sure that it does not contain antimicrobial residues. The primary control rests within the producers. First and foremost, they must ensure that milk from the treated animals does not enter the food chain during the prescribed WP. Controls are then carried out by milk processors (sometimes by milk producers or collectors), using rapid screening tests. When a bulk milk tank tests positive, the result is confirmed and quantified by a confirmative method. A bulk milk tank that exceeds the MRL must be removed.

1.1 AIM OF RESEARCH

The purpose of this study was to examine, on commercial farms, if treating clinical mastitis requires longer withhold period than the prescribed, as well as which particular screening test is suitable to use on a farm.

1.2 HYPOTHESIS

1. The excretion of drugs in milk of mastitic cows and cows with repeated mastitis is different compared to healthy cows.

2. Off-label use of antimicrobial substances, especially the use of different preparations simultaneously, may result in prolonged excretion of antimicrobials in milk.

3. Antimicrobial substances used for treatment of cattle cannot be detected by one screening test only.

2. LITERATURE REVIEW

The term *mastitis* in dairy cows means inflammation of the mammary gland, in the most cases caused by a microbiological agent. It is categorized as *clinical* or *subclinical* depending on whether visible signs are present or not, and *acute* or *chronic* mastitis based on the duration. Clinical mastitis is characterized with the presence of flakes or clots in milk, watery milk instead of normal consistency, oedema, painful on palpation and with increased temperature. Sometimes these signs are combined with general signs like hyperthermia, anorexia and impaired general condition of the animal (Gruet et al., 2001). During acute mastitis the signs are severe and appear rapidly, while at chronic mastitis the signs are mild and persist for a long time (Blowey and Edmondson, 1995).

Subclinical mastitis is present in most of the herds. Increased somatic cell count (SCC) in milk suggests subclinical mastitis. It is treated during the dry period, thus increasing the productive lifespan of the cows (Gruet et al., 2001). It is assumed that 70-80% of the yearly financial losses per cow caused by mastitis is due to reduced milk production from asymptomatic, subclinical mastitis (Gill et al., 1990).

Treatment at drying off has two major purposes. First, to treat intramammary infections, and second, to prevent new one at the beginning of the dry period. Most of the new infections appear during the first three weeks of the dry period and during the first month after parturition. The therapeutic efficacy of the treatment depends on the involved pathogens and on the treatment itself (Gruet et al., 2001).

2.1 MOST COMMON MASTITIS-CAUSING PATHOGENS

More than 130 microorganisms have been isolated from bovine mammary gland (Watts, 1988). The milking process is the most critical point where the uninfected udder quarters can be infected. Contagious bacteria spread generally during milking. This group includes: streptococci (*Streptococcus agalactiae*) and coagulase positive staphylococci (CPS, *Staphylococcus aureus*).

Most of the intramammary infections during the dry period, are caused by environmental bacteria. Environmental pathogens are ubiquitous and cows are exposed to these microorganisms for their entire life. They can invade cow udder during milking, between milkings and during the dry period. The most important pathogens in this group are: Enterobacteriaceae (*Escherichia coli, Klebsiella spp., Enterobacter spp., Serratia spp.* and *Proteus spp.*), Pseudomonadaceae (*Pseudomonas aeruginosa*), streptococci (*Streptococcus uberis*, *Streptococcus dysgalactiae*) and enterococci (*Enterococcus faecalis*). Environmental mastitis is mainly associated with clinical mastitis rather than subclinical mastitis (Gruet et al., 2001). *Mycoplasma spp.* unlike environmental and contagious pathogens, can spread from cow to cow through aerosols and invade the udder through bacteraemia (Erskine, 2016).

The most common bacteria to cause clinical mastitis are considered *Streptococcus uberis* and *Escherichia coli*, but mastitis caused by *Trueperella* (former: *Arcanobacterium*) *pyogenes* has the most unfavourable prognosis. There is no effective antimicrobial therapy against *Arcanobacterium pyogenes*, the infected quarter is damaged permanently, milk production is decreased, and the changes are advancing leading to atrophy of the mammary gland (Vasil, 2009).

2.1.1 Staphylococcus aureus

Staphylococcus aureus are haemolytic Gram-positive cocci. Most of the *S. aureus* strains convert the fibrinogen to fibrin by synthetizing an enzyme – coagulase, hence the term coagulase-positive staphylococci (Blowey & Edmondson, 2010). *S. aureus* and *Streptococcus agalactiae* are the two most important contagious bacteria among mastitis pathogens. This bacterium is not present on healthy, intact skin, but quickly colonize damaged skin and teat lesions. The infection occurs during milking, when milk contaminated with *S. aureus* comes in contact with a healthy udder, thus invading the teat canal. It produces toxins that destroy cell membranes and can directly damage the udder epithelium.

During infection with *S. aureus*, the bacteria firstly damage the tissues covering the teat and the gland cistern in the quarter, causing formations of scars. Then they penetrate deeper into the duct system and establish deeply rooted pockets of infection in the alveoli, the milk secreting cells.

This is followed by formation of abscesses that separate the bacteria from further spreading in the mammary gland, as well as preventing the immune system to react. Due to abscesses formation, antimicrobials are prevented from reaching the bacteria. Therefore, treating mastitis caused by *S. aureus* is very difficult. Another way of avoiding the antimicrobialsis by hiding into the neutrophils (white blood cells) and other host cells. Development of antimicrobial resistance against some beta-lactam antibiotics can additionally influence the outcome of therapy.

Infection with *S. aureus* is usually subclinical without evident changes in the milk or udder, but with increased SCC. Since the treatment is not very efficient, infected cows are generally separated from uninfected or culled. Preventing the conditions that support the spread of *S. aureus* in the herd is the best way to deal with this species. Prevention measures include improved hygiene, milking infected animals last, properly functioning milking equipment, antimicrobial treatment of infected animals, dry cow therapy, applying dry cow therapy in heifers, dry and clean conditions in the peripartum period (Petersson-Wolfe et al., 2010).

2.1.2 Streptococcus uberis

Streptococcus uberis is a Gram-positive, aerotolerant anaerobe with a cell wall structure similar to *Staphylococcus spp*. It is mostly present in the cow's environment, especially in straw bedding and straw yards. Unlike straw bedding, sand-based bedding does not support this bacterium (Kudi et al., 2009). The risk of infection with this bacterium increases during the early dry period and early lactation. Of the clinical cases, 56% originate in the dry period (Kromker, 2014). *S. uberis* causes clinical and subclinical infections. Mastitis caused by *S. uberis* is mainly acute, with a rapid onset, and fortunately, it is usually very responsive to a range of antimicrobials. Some strains can act as a contagious pathogen resulting in chronic mastitis cases. Keeping clean and dry environment accompanied with good milking practice is of utmost importance in preventing new infections (Kudi et al., 2009).

2.1.3 *Escherichia coli*

Escherichia coli is an environmental pathogen present in large number in the faeces. The species is isolated in 80% of the infections caused by coliforms. A lipopolysaccharide (LPS) component in the cell wall of Gram-negative bacteria is considered as main virulence factor in coliform bacteria that initiate the clinical signs in the animal. Infections with *E. coli* are most common cause of clinical mastitis in herds with low SCC. The clinical signs can vary from mild inflammation in the quarter, with minor changes in milk appearance without systemic signs, to severe clinical signs and highly decreased milk production (Suojala et al., 2013). The bacterium produces very strong toxins. Mastitis caused by this bacterium is usually acute with a general endotoxaemia and raised body temperature, loss of appetite and can be lethal if the cow is not treated (FAO, 1989). Cows infected during the puerperium can die and only 30-50% of them return in full lactation. Chronic infections are rare and insignificant for this bacterium. Fluoroquinolones and cephalosporins are confirmed as beneficial in the treatment of *E. coli* when given parenteral (Suojala et al., 2013). The severity of *E. coli* mastitis and the outcome is believed to be determined by cow factors rather than by *E. coli* pathogenicity (Burvenich et al., 2003).

2.1.4 *Streptococcus dysgalactiae*

Streptococcus dysgalactiae - a Gram-positive haemolytic bacterium is the third most common environmental bacterium behind *E. coli* and *Streptococcus uberis* (Blowey & Edmondson, 1995). In another article by the same authors *S. dysgalactiae* it is placed as the fourth major cause of contagious mastitis (Blowey & Edmondson, 2010). Basically, *S. dysgalactiae* can belong in both groups, since it is easily transmitted during the milking process and is ubiquitous. It is commonly found on teat skin, particularly when the skin is damaged. Mammary gland carriers are not so important. *S. dysgalactiae* is present on the tonsils and by licking could be transmitted to teats. This can be the reason of the common mastitis caused by *S. dysgalactiae* in heifers and dry cows (Blowey and Edmondson, 1995). The cure rate is high in the dry period, as well as during lactation. During the dry period the cure rate is nearly 100% (Whist et al., 2007).

2.1.5 *Streptococcus agalactiae*

Streptococcus agalactiae is a Gram-positive, beta-haemolitic species, producing very small colonies. It is highly contagious and is easily transmitted from cow to cow during the milking process. It causes subclinical and mild to moderate clinical mastitis in dairy cows. The cows with subclinical infection have an elevated SCC without abnormal milk. The highest concentration is in the udder, but it can also be found in the teat canal and the teat skin, especially if the surface is damaged (Blowey & Edmondson, 2010). Infection with *S. agalactiae* is followed by elevated SCC and total bacterial count, as well as decreased milk production and milk quality (Keefe, 1997). Unlike *S. aureus*, *S. agalactiae* is very responsive to antimicrobial therapy (Blowey & Edmondson, 2010).

2.1.6 Coagulase-negative staphylococci (CNS)

Nowadays, the genus *Staphylococcus* counts more than 50 species and subspecies, whereas around 10 are associated with bovine mastitis. Based on their ability to coagulate plasma, they are divided into coagulase-positive and coagulase-negative staphylococci. Most commonly isolated from milk are *Staphylococcus chromogenes* and *Staphylococcus simulans,* followed by *Staphylococcus hycus* and *Staphylococcus epidermidis*. CNS species in routine mastitis are treated evenly. Mastitis caused from CNS species, unlike *Staphylococcus aureus* and coliforms, is mild and usually subclinical. Infection with CNS can occur before calving and during lactation. Infected cows during lactation have increased SCC and decreased milk production. The prevalence of CNS in cows with either clinical or subclinical mastitis varies from country to country, partly because of the usage of different cell forming units (CFU) cut-off values. In Germany, the presence of CNS was found in 35% of the quarters with subclinical infection.

Treatment of CNS mastitis include antimicrobial therapy and non-antimicrobial means such as frequent milking. Frequent milking is preferred in some countries for treatment of subclinical and mild mastitis cases. CNS responds well to antimicrobial therapy with 80% to 90% efficacy. Penicillin G is effective and very often used against mastitis caused by these organisms (Pyörälä and Taponen, 2009).

2.1.7 *Klebsiella spp.*

Klebsiella spp. (Fam. *Enterobacteriaceae*) are also mastitis-causing coliform bacteria. From this genus, *K. pneumoniae* is the most common mastitis-causing pathogen in dairy cows followed by *K. oxytoca*. Like other coliforms, infection with *Klebsiella spp.* originates from organic matter including manure, wooden bedding, drinking water and feed. Outbreaks of *Klebsiella spp.* are often associated with sawdust bedding. The survival rate is much higher in case of *Klebsiella* mastitis compared to *E. coli* mastitis. Unlike *E. coli*, *Klebsiella spp.* infection lasts significantly longer and responds poorly to treatment. Higher prevalence of *Klebsiella* mastitis has been noticed in herds with low SCC (<150,000) than in herds with medium bulk milk SCC (150,000 – 250,000 cells/ml) (Munoz et al., 2006).

2.1.8 Other coliforms

Enterobacter* aerogens & *cloacae, ***Serratia marcesans***, ***Citrobacter*** together with *E. coli* and *K. pneumoniae* are common coliform bacteria that cause mastitis (Eberhart et al., 1979). All of them are normally present in the soil, digestive tract and manure. Concentrations higher than 1.000.000 cells/g in the bedding increase the chance of udder infection. After infecting, they either multiply rapidly or remain inactive. When destroyed by the immune system they release endotoxins that can cause many clinical signs associated with coliform mastitis, including high fever, loss of appetite, rapid weight loss, abnormal milk and decreased production (Maroney, 2005).

2.1.9 *Corynebacterium bovis*

Corynebacterium bovis is a contagious, facultatively anaerobic, Gram-positive bacterium. *C. bovis* causes only moderate increase in SCC. It colonizes the teat canal, whence it can spread to other cows by milking. Like many other bacteria, some post-milking teat disifectants (but not dodecylbenzene sulfonic acid) and dry cow therapy are very effective against this bacterium

(Petersson-Wolfe and Swartz, 2016).

2.1.10 Mycoplasma

Mycoplasma species have been associated with several cattle diseases, including: otitis media, urogenital tract inflamation, arthritis, pneumonia, and mastitis. *M. bovis* is the most common and most important mastitis causing agent among mycoplasma species, followed by *M. californicum* and rarely *M. bovigenitalium* and *M. alkalescens* (Fox, 2012). *M. bovis* and *M. californicum* are highly contagious with poor response to antibiotics. Usually most of the cows infected with these organisms have to be culled (Blowey and Edmondson, 2010).

2.1.11 Mycotic mastitis

Mycotic mastitis has been associated with more than 26 fungal species. Antimicrobial treatment, teat cannulas and syringes are common sources of infection with yeasts and molds, particularly contaminated treatment preparations (Ainsworth and Austwick, 1959). Unlike bacterial mastitis, there is no effective treatment against mycotic mastitis. The recovery is spontaneous, usually within 2 months, however, it can last up to 5 months. *Criptococcus neoformans* is most frequently isolated among the other fungi, causing also a serious form of mastitis (Swartz and Petersson-Wolfe, 2016).

2.2 MILK QUALITY ASSOCIATED WITH SOMATIC CELL COUNT (SCC)

Somatic Cell Count (SCC) is used as a main indicator of milk quality. Somatic cells are composed of leucocytes (75%) and epithelial cells (25%). Higher SCC indicates a possible infection and is followed by lower milk production. An udder with SCC level less than 200.000 cells/ml is considered physiologically normal. In EU the upper accepted limit of SCC in milk is

400.000 cells/ml. Unlike in the EU, in the United States this limit is 750.000 cells/ml. Another regulated parameter is bacterial counts (Plate Loop Counts, PLC) in milk, which has to be less than 100.000 per 1 ml. Larger farms had more antimicrobial violations and lower SCC and PLC. Farms with high SCC had higher PLC as well as more antimicrobial violations (van Schaik et al., 2002). The risk of antimicrobial violation is higher in herds with high SCC (Ruegg and Tabone, 2000). High SCC value indicates presence of subclinical mastitis, usually caused by *S. aureus* and *S. agalactiae* among the other potential pathogens.

2.3 MASTITIS

Intramammary infection (IMI) is considered the presence of the same pathogen in two of three consecutive cultures sampled at different milkings or the presence of a pathogen in both samples of duplicates sampled at the same time. Pathogens like *Streptococcus agalactiae* and *Staphylococcus aureus* have low rate of environmental contamination of milk samples hence, when they are isolated only once from the infected udder, it can be enough to determine the aetiology. Erskine et al. (2003) have not isolated any bacterium in 30 – 35% of milk samples collected from cows with clinical mastitis. However, this percentage is much lower in some others studies (Guterbock et al., 1993).

In the United States around 17% of dairy cows show signs of mastitis each year (Stockler et al., 2009). Nevertheless, the common method of determining the health of the udder is correlated with the SCC. Higher SCC of 200.000 cells/ml indicates a diseased udder/quarter, possibly a subclinical infection.

Holding to recommended farm practices (e.g. washing udders before milking, teat dipping after milking, regular maintenance of milking machines, proper treatment of clinical cases, antimicrobial treatment of all cows at drying off, removal of cows with chronic mastitis, milking infected cows last) has shown to be important in prevention and control of mastitis (Gill et al., 1990).

During mastitis, the level of lipase and plasmin enzymes in milk is increased leading to degradation of milk fat and casein, respectively (Blowey and Edmondson, 1995). Mastitis reduces the milk quality, hence lower milk price and shortened productive lifespan of the affected cows are consequences of mastitis. It subsequently causes significant economic losses (Rajala-Schultz et al., 1999; Petrovski et al., 2006; Halasa et al., 2007). The costs following mastitis are direct and indirect. Direct losses appear from discarded milk, drugs and veterinary costs. Indirect losses appear from decreased milk production during the remainder of the lactation as a result of udder damage and/or subclinical infection, penalties, lower milk quality (increased cell count), extra labour requirements, forced culling and death (Blowey and Edmondson, 1995).

Treatment of mastitis is the most frequent cause of drug use on dairy farms, and it constitutes a large portion of the total amount of the drugs used (Erskine et al., 2003). A treatment of clinical mastitis during lactation is considered a failure when there is not clinical improvement within 5 - 7 days (Gruet et al., 2001). Mastitis is treated intramammary and parenterally. Highly lipophilic drugs are used in the systemic treatment of mastitis such as macrolides, fluoroquinolones, and penethamate hydriodide (prodrug of Penicillin G), due to their ability to pass the udder epithelial barrier. The efficacy of parenteral treatment with penethamate hydriodid was not significantly different compared to intramammary treatment with cloxacillin/ampicillin combination regarding the clinical and bacteriological cure. Parenteral treatment seemed to affect also other quarters that might have been subclinically infected, thus lowering the SCC in the milk (Sérieys et al., 2005).

2.4 MASTITIS TREATMENT

Early detection of the infection and onset of treatment gives the best outcome (Barkema et al., 2006). Higher parity and higher SCC is associated with a lower chance of cure (Ziv & Storper, 1985; Owens et al., 1988). Infected hind quarters have lower chance of cure than the front quarters. (Sol et al., 1994).

Mastitis can be treated during the *lactation period* or during the *dry period*. The approach of treatment differs between the *mild* and *severe clinical mastitis* in the lactation period. Severe clinical mastitis include systemic signs, whilst mild clinical mastitis is usually accompanied by abnormal milk with or without local inflammation of the affected quarter.

An ideal antimicrobial for systemic mastitis therapy suggested by Ziv (1980) should have the following characteristics:

- low minimal inhibitory concentration (MIC) against the majority of udder pathogens,
- high bioavailability from intramuscular injection sites,
- weakly basic and nonionized in serum (close to serum's pH),
- be lipid soluble,
- low degree of protein binding,
- a long half-life in the body,
- retain activity in inflammatory secretions, and
- no drug accumulation in specific organs.

In most dairy herds the most common cause of severe mastitis are the coliform organisms as well as the main cause of death or agalactiae. Hence, the primary therapy for severe clinical mastitis should target coliform organisms. Nevertheless, other bacteria among coliforms should also be considered. Experimental infections with coliforms often resulted in severe clinical mastitis. They usually retreat spontaneously, not more than 10 days from the infection. However, the resulting inflammation and leukocytosis in the affected quarter may continue for the next few weeks or the quarter may become agalactic, despite the absence of bacterial culture in milk. Three intramuscular doses of cefquinome (IV generation cephalosporin) improved clinical outcome of experimentally infected cows with coliforms. Intravenous oxytetracycline also showed good results in cows with clinical coliform mastitis. Good results were achieved after intramuscular administration of ceftiofur sodium (III generation cephalosporin) in naturally infected cows with coliform organisms.

Nevertheless, in severe cases of mastitis, when the cause-agent is unknown, Gram-positive organisms should also be targeted intramammarily (Erskine et al., 2003).

A therapeutic regimen for severe clinical mastitis include:

- supportive therapy (fluids), especially in cows showing signs of shock;
- maintain effective concentrations of antibacterial drugs in plasma;
- using antibacterial drugs with broad spectrum of activity; and
- administering intramammarily antibacterial drugs targeting Gram-positive IMI, unless analysis indicate a Gram-negative or mycotic infection.

Unlike severe clinical mastitis, treatment of mild mastitis is a more voluntary therapeutic decision and can be lengthened until identification of the pathogen. When Gram-positive cocci are isolated, treatment is recommended. Sometimes premature agalactiae in chronically infected quarters is practiced as an alternative to culling the cow, especially when the therapy is not effective. This often results in compensatory production in the remaining three quarters, similarly to that gained from four quarters.

Subclinical infections are often caused by the contagious pathogens *Staphylococcus aureus* and *Streptococcus agalactiae*. Many subclinical IMI are chronic. Unlike mild clinical mastitis, subclinical mastitis does not require immediate treatment. It is usually treated in the dry period (Erskine et al., 2003).

2.5 DRUG PHARMACOKINETICS IN THE MAMMARY GLAND

Intramammary treatment of mastitis with antibiotics is probably more effective against non-invasive bacteria such as *Streptococcus agalactiae* and coagulase-negative staphylococci, but is less effective against bacteria that are able to invade the udder tissue deeper or create abscesses, such as *Staphylococcus aureus*, *Streptococcus uberis* and some coliform bacteria. Hydrophilic

drugs distribute well through the central udder compartment but not in the deeper udder tissue. In contrast, by passive diffusion only nonionized and unbound lipophilic drugs can distribute further into the deeper udder tissues and into the plasma (Gehring and Smith, 2006).

The normal pH of milk is slightly acidic (pH 6.4-7.0), while the pH of plasma is 7.4. During mastitis the milk pH increases, thus the pharmacokinetic properties of the drugs are changed (Gehring and Smith, 2006). Elimination of drug residues from milk after intramammary treatment occur by diffusion of the drug into the plasma or by milking. Residues pass the epithelial barrier most likely by passive diffusion. Only unbound, nonionized and lipid soluble drugs are able to pass through the udder epithelia into the plasma (Ziv and Sulman, 1975; Gehring and Smith, 2006).

Both, lipid solubility and dissociation constant are important for the absorption rate of the drug from the udder compartment. Due to rapid absorption, highly lipophilic drugs deplete faster from the milk. Some antibiotics have higher affinity to protein binding than others. This nonspecific binding forms - drug-protein complexes are too large to cross the udder epithelial barrier. However, this interaction is insignificant regarding the excretion of antibiotics from the udder. Nor the interaction between antibiotics influences the excretion rate of antimicrobials from the udder. Comprehensive tissue binding, typical for aminoglycosides (dihydrostreptomycin, neomycin, kanamycin), can cause uneven and restricted drug distribution (Ziv and Sulman, 1975).

Penicillins absorbed faster than tetracyclines, as well as the poorly lipid-soluble basic aminoglycosides. In the same study, no significant difference was noticed in the absorption rate from the mammary gland between antibiotic mixtures and individually administered antibiotics, thus supporting the hypothesis of passive diffusion (Ziv and Sulman, 1975).

2.6 DRUG RESIDUES IN MILK

Milk, together with dairy products, constitutes a large portion of the average daily intake per capita in Europe (more than 150 kg milk per capita/year). It is also an indispensable nutrient for normal development throughout life especially in younger age (IFCN, 2006; Weaver et al., 2013).

Administered veterinary drugs may be excreted from the animal unchanged. Once excreted with manure and urine, they can contaminate the land by the manure applied on the croplands. Drugs can remain in the soil for a certain time before being degrade, which can eventually end up in potable water (Kumar et al., 2005).

The presence of antimicrobial residues in milk are unwilling since the potential detrimental effect that they have on people allergic to antibiotics, inhibition of starter cultures used by the milk industry and initiating antibiotic resistant bacteria (Seymour et al., 1988). Among antibiotics, penicillins in milk are the most important allergens. The presence of tetracyclines and aminoglycosides in milk can cause immunologically-mediated reactions, but they are not considered so clinically hazardous as penicillins (Dewdney and Edwards, 1984).

Mastitis treatment and dry cow therapy is most commonly associated with antimicrobial residues in milk (Booth & Harding, 1986). After off-label usage of drug preparations, the WP should be long enough for the antimicrobial residues to fall below the MRL (Smith et al., 2005). However, antimicrobials are occasionally detected in bulk milk tanks.

Each country in the EU has its own national legislation in accordance with the EU Directive 2001/82/EC on veterinary medicinal products for off-label use of antimicrobial drugs. Slovenian national legislation for off-label use of antimicrobial preparations intended for food producing animals has WP not less than 7 days (14 milkings) in lactating cows (Uradni List RS, 17/14;).

Off-label treatment is practiced in Slovenia and elsewhere. It is a general practice, since better cure rate is achieved when the treatment is prolonged or the dosage is increased (Pengov et al., 2001; Gillespie et al., 2002; Oliver et al., 2003; Oliver et al., 2004a, 2004b; Kasravi et al., 2011).

In the study of Lucas et al. (2009) after treating bovine subclinical mastitis via the intramuscular route, higher presence of azithromycin (macrolide) in the milk from mastitic udder quarters was observed. It was assumed that lower milk production and mammary health status contributed to the prolonged excretion of azithromycin. Improper storage conditions of drug preparations can also lead to prolonged drug excretion due to changes in the drug composition (Knappstein et al., 2005). Different pharmacokinetic properties of norfloxacin were noted after intramuscular treatment of lactating cows suffering from clinical and subclinical mastitis (Gips and Soback, 1999). There was no significant difference in the pharmacokinetics of cephapirin (I-generation cephalosporin) after intramammary infusion in cows milked 2 and 3 times per day. Cows with lower milk production absorbed more of the drug than high productive cows and had a longer mean residence time. In this study healthy cows were used (Stockler et al., 2009).

Gentamicin, an aminoglycoside, was not detected in plasma after intramammary administration in healthy cows, but was detected in plasma after treatment of mastitic udder quarters. No crossover of gentamicin was noted in the untreated quarters after intramammary treatment of either the infected or uninfected udder quarters (Sweeney et al., 1996).

Elimination of cefquinome was more dependent on the influence of individual cows, whereas, in the case of penicillin, with the increasing of milking frequency the excretion time increased and vice versa (Knappstein et al., 2003). Seymour et al. (1988) noted that intramuscular or intrauterine applied penicillin G and intramammary applied cephapirin are more prone to prolonged excretion than other antimicrobials.

In a study performed in Michigan, mastitis treatment was responsible for 92,7% of the antimicrobial violations in bulk milk tanks, whereas 30% came from dry cow treatments (Mellenburger, 1998). Farmer survey showed that 12-17% of the violations were due to short WP (Booth 1982; Mellenburger, 1998). In Table 1 are shown the common causes for bulk milk contamination in 1981 quoted by Booth (1982), however these relative figures are likely to have changed ever since. They are generally based on farmer opinion instead of proven causes.

Table 1. Reasons suggested for antibiotic test failures (Booth 1982).
Preglednica 1. Najpogostejši vzroki, zaradi katerih je bilo mleko pozitivno na antibiotike (Booth 1982).

Reason	Percentage
Poor records, or none	32
Not withholding milk for full period	32
Calving early/short dry period	15
Prolonged excretion	12
Contamination of recorder jars	9
Withholding milk from treated quarter only	8
Lack on advice on withholding period	8
Mechanical failure	6
Recently purchased cows	3
Milking through jars	1
Use of dry-cow preparation during lactation	1

*The figures total more than 100 as some respondents gave more than one reason.

The increased number of intramammary infusions was significantly associated with an increased risk of antimicrobial residues in milk after the withholding time. These animals were at 3,85 times higher risk than animals treated according to label instructions (McEwen et al., 1992). Milking frequency also affects the rate of drug elimination in milk following intramammary administration (Knappstein et al., 2006). The concentration of drugs in milk after intramammary treatment is also affected by the absorption rate of the drug from the udder epithelia (Moretain & Boisseau, 1989), which depends on the lipid solubility of the undissociated molecule. Erythromycin is rapidly absorbed and enter the systemic circulation following intramammary administration (Bajwa et al., 2007).

Change in elimination kinetics are not restricted to antimicrobials only. Elimination kinetics of other anti-inflammatory drugs are also affected. Elimination of flunixin – Nonsteroidal Anti-

Inflammatory Drug (NSAID) from milk differed in healthy and mastitic udder quarters. Violative residues of flunixin have often been reported in beef and dairy cattle (Kissell et al., 2015). The systemic elimination half-life was significantly longer for carprofen (NSAID) in mastitic cows (Lohuis et al., 1991).

Before the milk is placed on the market or sent for further processing, the producers in the dairy industry must be sure that it does not contain antibiotics above the MRL. The primary control rests with the producers. First and foremost, they must ensure that the milk from the treated animals does not enter the food chain during the prescribed WP. Controls are then carried out by milk processors (sometimes by milk producers or collectors), using screening tests. In the case when a bulk milk tank tests positive, the result is confirmed and quantified by a confirmative method. A bulk milk tank that exceeds the MRL values must be removed, and certainly it must not be used for human consumption.

The prevalence of antimicrobial positive bulk milk tanks varies between countries. The prevalence of positive bulk milk tanks in developed EU countries is lower than the other European and Non-European Countries. Detection of traces of antimicrobial residues (tetracycline, penicillin G, amoxicillin) in milk is very common, however concentration above MRL is unacceptable (Ghidini et al., 2002; Adesiyun & Webb, 1997; Adesiyun & Stoute, 2007; Alomirah et al., 2007; Khaskheli et al., 2008; Dimitrieska-Stojkovic et al., 2011; Bilandžić et al., 2011; Grădinaru et al., 2011; Moghadam et al., 2016; Aalipour et al., 2015; Rama et al., 2017).

2.7 ANTIMICROBIAL RESISTANCE – AN EMERGING ISSUE

Antimicrobial therapy remains essential for treating and in some cases preventing bacterial diseases. The development of resistance to animal antimicrobials is a threat to humans, because some bacteria can cause a disease in humans after they are transmitted via contaminated food.

Additionally, these bacteria are carrying genes that can be transferred to other pathogenic human bacteria (McKellar, 1998).

Infections caused by opportunistic bacteria such as methicillin-resistant staphylococci - MRS, including *Staphylococcus aureus* – MRSA and *Staphylococcus pseudintermedius* – MRSP, extended-spectrum beta-lactamase – ESBL producing Enterobacteriaceae (*E. coli*) and *Acinetobacter baumannii* have become a major challenge in human as well as in veterinary medicine (Walther et al., 2016).

Antimicrobial resistance can be induced in most mastitis pathogens, thus it decreases success of bacterial pathogen eradication or control. Guidelines for prudent use of antimicrobials in veterinary medicine (EC, 2015) have been published, however, they are not mandatory and mainly depend on acceptance by veterinarians and their clientele (Oliver SP et al., 2011). Implementing guidelines for prudent use of antimicrobials in order to minimize the development of resistance in veterinary medicine has greatly decreased their use. Some of the adoptions include:

- less prescriptions and a marked reduction of treatment days,
- shifting from older preparations with high rate of inaccurate dosing (generally underdosing) to more modern and precise dosed preparations,
- tendency towards more therapeutic indications rather than prophylaxis (Ungemach et al., 2006).

Organic farming had lower prevalence of antimicrobial resistant bacteria than conventional farming, upon shifting from conventional to organic management. CNS were significantly less resistant to β-lactams when isolated from milk after the herd switched to organic management (Park et al., 2012). Similar finding was noted in the study of Suriyasathaporn (2010), where after changing from conventional to organic dairy farming less antimicrobial resistant bacteria were found after 6 months from the transition. Decreased resistance to ampicillin and streptomycin was significant on the organic farm system.

Excessive use of cloxacillin and oxacillin in mastitis treatment showed higher percentage of resistant staphylococci and streptococci compared to normal use (Suriyasathaporn et al, 2012).

Antimicrobials in dairy cattle may contribute to increased antimicrobials resistance, however, there is no evidence that emerging resistance among mastitis pathogens origin from the treatment of mastitis, even though many of these antibiotics have been used in the dairy industry (Oliver & Murinda, 2012).

2.8 ANTIMICROBIALS PERMITTED FOR USE IN DAIRY CATTLE (37/2010, EC)

2.8.1 Tetracyclines

The tetracycline group of antibiotics counts around 10 members of which oxytetracycline, chlortetracycline, and demethylchlortetracycline occur naturally. Chlortetracycline and oxytetracycline were the first members of the tetracycline group of antibiotics discovered in the late 1940s as products of *Streptomyces aureofaciens* and *S. rimosus*, respectively. A few years later tetracycline was synthesized from chlortetracycline (Nelson and Levy, 2011). Tetracyclines exhibit a broad spectrum of activity against Gram-negative and Gram-positive bacteria, as well as some other types of microorganisms such as chlamydia, mycoplasmas, rickettsia, and protozoan parasites. They inhibit protein synthesis by reversible binding to the 30S ribosomal subunit, specifically at the aminoacyl-tRNA acceptor ("A") site on the mRNA ribosomal complex preventing ribosomal translation (Speer et al., 1992; Brodersen et al., 2000; Chopra and Roberts, 2001). Soon after their discovery, oxytetracycline and chlortetracycline started being used as growth promoters in some countries (USA, Australia, United Kingdom) (Chopra and Roberts, 2001), and nowadays, oxytetracycline is also used in plant agriculture in the USA (Kumar et al., 2005). Tetracyclines can cause discoloration of teeth and depression of skeletal growth and are therefore not recommended for children up to the age of 6 to 8 years and for pregnant women (Speer et al., 1992; Navratilova et al., 2009). The absorption of tetracycline in the intestines is being

reduced by the calcium molecules which is known to form nonabsorbable chelates (Chopra and Roberts, 2001).

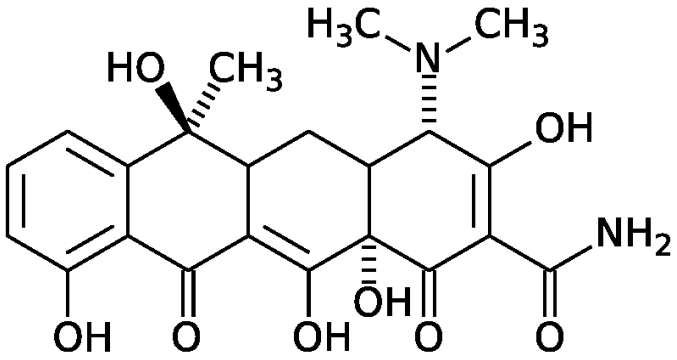


Figure 1. Molecular structure of tetracycline (Valentin et al., 2009).
Slika 1. Molekularna struktura tetraciklina (Valentin et al., 2009).

In order to protect public health and prevent any adverse effects, MRL values for pharmacologically active substances have been set down in the EU. For tetracycline, oxytetracycline, and chlortetracycline, together with their epimers, the MRL in milk is 100 µg/kg. The MRL has not been established for doxycycline since its use is prohibited in animals whose milk is intended for human consumption (EC, 37/2010).

After the Swann's report in 1969 on the use of antibiotics associated with the development of antimicrobial resistance, some antibiotics used as growth promoters were withdrawn and, in 2006, all antibiotics used in subtherapeutic levels in the EU were banned (Hao et al., 2014).

2.8.2 Beta-lactams

The beta-lactam antibiotic group include penicillins, cephalosporins, monobactams, and carbapenems. They all have a beta-lactam ring (Figure 2) necessary for antibacterial activity. In the treatment of mastitis the most widely used beta-lactam antimicrobials are the members of penicillins and cephalosporins. The beta-lactam antibiotics interfere with the transpeptidation process that links the individual peptidoglycan components of the bacterial cell wall. Beta-lactam antibiotics bind to and inactivate enzymes (transpeptidases, carboxypeptidases, endopeptidases, and transglycosidases termed as penicillin-binding proteins - PBPs) located on the inner surface of

the bacterial cell membrane (Oates et al., 1988). PBPs are enzymes involved in the process of cell-wall synthesis during growth and replication (Oates et al., 1988; Hornish & Kotarski, 2002).

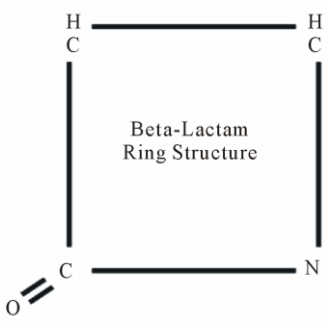


Figure 2. Structure of beta-lactam ring (Saini & Bansal, 2012).
Slika 2. Struktura beta-laktamskega obroča (Saini & Bansal, 2012).

2.8.2.1 Penicillins

Penicillin was accidentally discovered by Alexander Fleming in 1928 when his plates inoculated with *Staphylococcus* colonies were contaminated with moulds. In those days it was not able to extract enough penicillin for the experiments, so the use of penicillin as a therapeutic agent for treating infections became possible 12 years later, in the 1940s. Since then efforts were made to semi-synthesize other compounds from the penicillin's nucleus – 6-aminopenicillanic acid (6-APA) with better characteristics. Methicillin was the first semi-synthetic compound unaffected by penicillinase. Later in 1967 carbpenicillin was introduced, a penicillin analogue effective against *Pseudomonas aeruginosa* (Wright, 1999). Frequently used penicillins for treatment of mastitis are procaine benzylpenicillin (penicillin G), amoxicillin, ampicillin, and cloxacillin (Royster et al., 2015).

Amoxicillin and ampicillin are aminopenicillins, the first group of penicillins that were effective against Gram-negative bacterial strains, whilst, amoxicillin being effective against *E. coli* and *Klebsiella spp* (Wright, 1999; Royster et al., 2015). Ampicillin was created by adding an amino group to the basic benzylpenicillin molecule. Later were synthesized other aminopenicillins (Wright, 1999).

Penicillins have been widely used in the veterinary medicine. Their toxicity is low, only extremely high doses can initiate toxic effect. However, hypersensitive reactions are far more common with an estimated 3 to 10% of the general population are allergic to penicillin. Patient allergic to one penicillin member should be considered allergic to the other members (Wright, 1999). Much higher concentration of penicillin is necessary to sensitize a person than the concentration needed to trigger an allergic reaction in an already sensitized individual. Also, it takes much higher oral dose to cause an allergic reaction than parenterally administered drug. Therefore, it can be concluded that it is unlikely small concentrations of penicillins potentially present in food products of animal origin to sensitize a consumer, however, it is more likely to trigger a reaction in already sensitized individuals (EMA, 2008).

Penicillin can inhibit starter culture and delay acid production in dairy industry in very low quantities, as low as 0.006 µg per gram (0.01 IU).

In order to protect the consumers' health and secure dairy production, MRL for penicillins have been established (Table 2).

Table 2. Maximum residue limits of penicillins in milk and edible tissues (EC, 37/2010).
Preglednica 2. Najvišje mejne vrednosti ostankov penicilinov v mleku in užitnih tkivih (EC, 37/2010).

Penicillin	**Milk µg/kg**	**Edible tissues µg/kg**
Benzylpenicillin	4	50
Ampicillin	4	50
Amoxicillin	4	50
Nafcillin	30	300
Oxacillin	30	300
Cloxacillin	30	300
Dicloxacillin	30	300

2.8.2.2 Cephalosporins

Other group of beta-lactams was isolated from a strain of *Cephalosporium acremonium* with 7-aminocephalosporinic acid (7-ACA) nucleus. Using 7-ACA nucleus as the precursor, several generations of cephalosporins with broad-spectrum activity have been synthesized (Kong et al., 2010). Likewise the penicillins and the rest of the beta-lactam family, the beta-lactam ring is crucial

for antibacterial activity of cephalosporins. Cephalosporins generally are active against many Gram-positive aerobic bacteria, some Gram-negative aerobic bacteria and some anaerobic bacteria. They are mainly classified according to their relative *in vitro* spectrum of activity and structural similarities as first- (narrow spectrum), second- (expanded spectrum), third- (broad spectrum) or fourth-generation (extended spectrum) cephalosporins. Ceftiofur (third-generation) and cefquinome (fourth-generation) were developed solely for veterinary use. For lactating cows a few cephalosporins from first- and second-generation are approved worldwide strictly for treatment of mastitis (Hornish & Kotarski, 2002). In Table 3 are listed MRLs for cephalosporins permitted for use in dairy cattle.

The efficacy of three intramammary preparations containing cephalosporins were analysed after treatment of clinical mastitis in dairy cattle. One preparation contained combination of cephalexin (first generation cephalosporins) and kanamycin, the second preparation contained cefquinome (fourth generation cephalosporins) and the third cefoperazone (third generation cephalosporins). Mastitis treated with cephalexin & kanamycin and cefquinome showed better results than mastitis treated with cefoperazone (Bradley & Green, 2009).

Table 3. Maximum residue limits of cephalosporins in milk (EC, 37/2010).
Preglednica 3. Najvišje mejne vrednosti ostankov cefalosporinov v mleku (EC, 37/2010).

Cephalosporin member	Generation	Milk µg/kg
Cefacetrile	first	125
Cefalexin	first	100
Cefalonium	first	20
Cefapirin	first	60
Cefazolin	first	50
Cefoperazone	third	50
Ceftiofur	third	100
Cefquinome	fourth	20

2.8.3 Aminoglycosides

Streptomycin was the first identified aminoglycoside (in 1952 this discovery was awarded with Nobel Prize) isolated from *Streptomyces griseus* in 1944 (Schatz et al., 1944), followed by

neomycin isolated in the same laboratory from *Streptomyces fradiae* (Waksman et al., 1949). Kanamycin was isolated in 1957 from *Streptomyces kanamyceticus* (Umezawa et al., 1957) and gentamicin in 1963 from actinomycete *Micromonospora purpurea* (Weinstein et al., 1963).

Aminoglycosides are used for treatment of serious infections caused by Gram-negative bacteria, including *E. coli*, *Enterobacter*, *Pseudomonas* and *Salmonella* species, Gram-positive infections caused by *Staphylococcus* and some streptococci, and also against infections caused by mycobacteria (Hermann, 2007). In treating infections, aminoglycosides are usually combined with beta-lactam antibiotics (ampicillin) for achieving better synergistic effect against broad spectrum of bacteria, particularly when the causative agent is unknown (Dressel et al., 1999). All aminoglycosides have affinity for the bacterial 30S subunit of rRNA. By binding to the small rRNA subunit they interfere the protein synthesis resulting with synthesis of a defective protein (Davies, 2006).

Nevertheless, aminoglycosides have side effects. They are nephrotoxic and can provoke acute dose-dependent kidney failure. They can also induce permanent hearing loss (cochleotoxicity) and/or balance disorders (vestibulotoxicity) (Jiang et al., 2017).

Out of the members of aminoglycoside family, streptomycin (dihydrostreptomycin), neomycin, kanamycin, and gentamicin can be used in treatment of infections in dairy cattle. The MRL of the aminoglycosides in milk are presented in Table 4.

Table 4 Maximum residue limits of aminoglycosides in milk (EC, 37/2010).
Preglednica 4. Najvišje mejne vrednosti ostankov aminoglikozidov v mleku (EC, 37/2010).

Aminoglycoside member	**Milk µg/kg**
Dihydrostreptomycin	200
Streptomycin	200
Neomycin	1500
Kanamycin	150
Gentamicin	100

2.8.4 Sulfonamides

Sulfonamide (sulphonamide) is a term used as a generic name for the derivatives of para amino benzene sulfonamides. Sulfonamides are the first synthetic antimicrobials synthetized in the early 30s of the 20th century from prontosil (*Prontosil rubrum*) – an azo-dye with sulfonamide structure (Tacic, 2017). The general formula of sulfonamides is RSO_2NH_2, with SO_2NH_2 being the functional group. Sulfonamides express bacteriostatic activity against Gram-positive, Gram-negative organisms, some protozoa, fungi and *Chlamydia* genus, but they are ineffective against *Pseudomonas aeruginosa* and *Serratia spp* (Lavanya, 2017; Tacic, 2017). They inhibit para-aminobenzoic acid (PABA) necessary for the synthesis of folic acid, which is essential for the synthesis of DNA and RNA. Thus, sulfonamides delay bacterial growth and cell division (Lavanya, 2017).

Sulfonamides when combined with trimethoprim exhibit bactericidal effect. In 4-6% of treated patients sulfonamides cause side effects in form of hypersensitivity (most common reaction), fever, anaphylactic shock, serum sickness, systemic vasculitis, pneumonia hepatitis, myocarditis, interstitial nephritis, blood dyscrasia, and different skin reactions (Tacic et al., 2017). To protect public health MRL for all sulfonamides combined in milk should not exceed 100 μg/kg (EC, 37/2010).

2.8.5 Quinolones

Quinolones are synthetic drugs, synthesized from nalidixic acid (naphthyridine) which was isolated in 1962 by George Lesher and co-workers (Aldred et al., 2014). Since then 4 generations of quinolones have been synthesized by modification of the quinolone nucleus. These modifications changed the antimicrobial activity, pharmacokinetics, and metabolic characteristics (Andriole, 2005). In human medicine quinolones are one of the most commonly prescribed antibacterials for treatment of various bacterial infections. Unsurprisingly, the number of quinolone-resistant bacteria is increasing since the 1990s.

Quinolones are used to treat a wide range of bacterial infections caused by Gram-negative and some Gram-positive organisms. In the bacterial cell, quinolones act on gyrase and topoisomerase IV enzymes changing them into toxic enzymes that disintegrate the bacterial chromosome (Aldred

et al., 2014). Enrofloxacin is often used for treatment of mastitis caused by *E. coli*. Dairy cows with *E. coli* mastitis had lower SCC after enrofloxacin treatment than untreated cows (Presson et al., 2015).

From the quinolone class, enorofloxacin with its metabolite ciprofloxacin, danofloxacine, marbofloxacin, and flumequine can be used in dairy cattle, either for mastitis treatment or other diseases. Their MRLs are given in Table 5.

Table 5. Maximum residue limits of quinolones in bovine milk (EC, 37/2010).
Preglednica 5. Najvišje mejne vrednosti ostankov kinolonov v mleku (EC, 37/2010).

Quinolone member	**Milk µg/kg**
Enrofloxacin + ciprofloxacin	100
Danofloxacine	30
Marbofloxacin	75
Flumequine	50

2.8.6 Macrolides and lincosamides

In 1960s spiramycin was the first macrolide used in animals for food production, whilst in 1970s erythromycin and tylosin were introduced. From the lincosamides, pirlimycin and lincomycin are used in dairy cattle. In food animals in some or all EU member states, by 2013, eight macrolides and two lincosamides were authorised.

Macrolide and lincosamide antimicrobials hinder the bacterial synthesis by binding to 50S subunit of the ribosome. Due to the similar mechanism of action of both groups, resistance is usually linked. Macrolides and lincosamides are active against many Gram-negative and Gram-positive bacteria, as well as some anaerobes such as *Fusobacterium*, *Clostridium* and *Bacteroides* spp. Unlike macrolides, lincosamides are not effective against *Enterococcus faecalis* and have decreased activity against Pasteurellaceae.

Semi-synthetic macrolides have a very long half-life (tulathromycin 4 days in cattle and pigs; gamithromycin less than 2 days in cattle, tildipirosin 9 days in cattle). When administered parenterally, severe tissue irritation with pain and inflammation is a common issue of all

macrolides. Macrolides are very often combined with colistin or aminoglycosides, as well as sulfonamides, trimethoprim, oxytetracycline or ampicillin.

The MRLs for macrolides and lincosamides used in animals whose milk is intended for human consumption are shown in Table 6.

Table 6. Maximum residue limits of macrolides and lincosamides in milk (EC, 37/2010).
Preglednica 6. Najvišje mejne vrednosti ostankov makrolidnih antibiotikov in linkosamidov v mleku (EC, 37/2010).

Macrolides	**Milk µg/kg**
Erythromycin	40
Spiramycin	200
Tilmicosin	50
Tylosin	50
Lincosamides	
Pirlimycin	100
Lincomycin	150

2.8.7 Other antimicrobials permitted for use in dairy cattle

Thiamphenicol is a chloramphenicol analogue with a broad-spectrum antibiotic activity including anaerobes. Thiamphenicol is used in cattle and poultry for the treatment and control of respiratory and intestinal infection, administered by oral route or intramuscular route. Thiamphenicol is also used intramammarily for mastitis treatment during lactation and during the dry period.

Thiamphenicol inhibit bacterial growth by binding to the 50S subunit of the bacterial ribosome and blocking the enzymatic function of peptidyl transferase. Unlike chloramphenicol, there is no data that show risk for aplastic anemia in treated human patients with thiamphenicol, however, a reversible dose-dependent bone marrow depression is noted.

Based on the most sensitive bacterial species from the human gut flora – *Fusobacterium spp*., a microbiological ADI of 2.5 µg/kg (150 µg for an adult person of 60 kg) has been established. Out of this value a MRL for thiamphenicol in food-producing species has been calculated. The MRL

for thiamphenicol in milk is estimated at 50 μg/kg, the same value has been estimated for muscle tissue, fat, liver and kidney (EMEA/CVMP/162614/2006-Final; EMEA/MRL/256/97-Final).

Bacitracin is a metalopeptide antibiotic produced by *Bacillus subtilis* and *Bacillus licheniformis*. It has been discovered in 1943 and widely used since then. Despite the wide use of bacitracin in the past several decades, bacterial resistance is still rare. For its bactericidal activity bacitracin requires a divalent Zn^{2+} ion, while being able to attach other transition metal ions as well, including Mn^{2+}, Co^{2+}, Ni^{2+} and Cu^{2+}. It is effective mainly against Gram-positive organisms. Bacitracin likewise the β-lactam antibiotics inhibit the synthesis of bacterial cell wall (Ming & Epperson, 2002).

Bacitracin is barely absorbed after oral applications, up to 95% is excreted via faeces. In humans it is intended for topical use only, given the nephrotoxic activity. In veterinary medicine, bacitracin is used for treatment of mastitis in lactating cows, in combination with tetracycline, neomycin and prednisolone. The microbiologically based ADI in humans is 3.9 μg/kg body weight and 234 μg/day for an adult person. The MRL in bovine milk deducted from ADI is 100 μg/kg for the sum of bacitracin A, bacitracin B and bacitracin C (EC, 37/2010).

Trimethoprim is often used in veterinary practice in combination with a sulfonamide (often sulfamethoxazole). It is active against Gram-positive and Gram-negative bacteria including *E. coli*, *Klebsiella*, *Proteus* and *Staphylococcus* species. It inhibits DNA synthesis in bacterial or protozoal cell by blocking the synthesis of folinic acid from folic acid. Trimethoprim is not recommended for women during pregnancy because of the teratogenic effect noted in the rat. The MRL for trimethoprim in bovine milk is set up as low as possible at 50 μg/kg (EC, 37/2010).

Clavulanic acid was firstly isolated in 1971 from *Streptomyces clavuligerus*. Clavulanic acid is a β-lactam inhibitor with a β-lactam structure similar to that of penicillins (Saudagar et al., 2008). It is usually combined with other antimicrobials (β-lactam) and in veterinary medicine is always combined with amoxicillin in a ratio of 1/4; clavulanic acid/amoxicillin trihydrate. It is used intramammarily in lactating cows for treatment of mastitis. The MRL of clavulanic acid in bovine

milk (200 µg/kg) is based on toxicological ADI (0.05 mg/kg^{-} body weight; 3 mg/person) because of the lower value than the microbiological ADI (EMEA, 2001).

Novobiocin is a natural product isolated from *Streptomyces niveus*. It consists of 3 parts: the sugar noviose, a coumarin moiety and a benzoic acid derivative. It is mainly active against Gram-positive bacteria and some Gram-negative (*Klebsiella* and *Proteus* strains). Novobiocin prevents DNA replication of the bacterial cell by inhibiting the DNA-gyrase. In 1000 fold higher concentration same action occurs in the mammalian cell. Novobiocin has been widely used in human medicine and also in food producing animals solely for intramammary treatment of lactating and dry cows. The ADI and MRL in bovine milk for novobiocin is 75 µg/person and 50 µg/kg, respectively (EMEA, 1999).

Colistin or *polymixin E* is a cyclopeptide antibiotic discovered in the 40s of the 20th century as a product of *Bacillus polymyxa* var. *colistinus*. It is effective against Gram-negative bacteria in which it interacts with the lipopolysaccharide (LPS) component of the outer membrane causing increased permeability of the cell membrane, leakage of cell contents and eventually cell death. However, other targets might be also involved (Suhren & Knappstein, 2005). Its MRL in milk is 50 µg/kg (EC, 37/2010).

Monensin (MRL in milk 2 µg/kg) is a polyether antibiotic with ionophoric activity produced by *Streptomyces cinnamonensis*. Despite its antimicrobial properties against Gram-positive bacteria, monensin has also been used as growth promoter in beef production until 2006. Today monesin is used to prevent ketosis in dairy cattle (EMEA, 2007).

All antimicrobial preparations for treating bovine mastitis that were and still are registered for use in Slovenia are gathered in Table 7 and 8.

Table 7. Intramammary preparations for treating bovine mastitis during lactation registered in Slovenia (Uradni list RS, 13/2003; Uradni list RS, 17/2014).
Preglednica 7. Pripravki za intramamarno dajanje za zdravljenje mastitisa med laktacijo, ki so registrirani v Sloveniji (Uradni list RS, 13/2003; Uradni list RS, 17/2014).

No	Drug	Composition	Manufacturer	Withhold period*
1	AMOXIKLAV®	200 mg amoxicillin trihydrate 50 mg clavulanic acid 10 mg prednisolone acetate in 10 ml oily suspension	LEK, tovarna farmacevtskig in memičnih izdelkov, d.d., Ljubljana, Slovenija	Milk – 4 milkings Meat – 7 days
2	AMPICLOX® L.C.	75 mg ampicillin sodium 200 mg cloxacillin sodium in 3 g oily suspension	Pfizer Animal Health S.A. Italy	Milk – 5 milkings Meat – 7 days
3	AMPIVET® K forte	75 mg ampicillin sodium 200 mg cloxacillin sodium in 5 ml suspension	VETERINA d.o.o., Croatia	Milk – 6 milkings Meat – 7 days
4	AMPIVET® K	200 mg ampicillin trihydrate 100 mg cloxacillin sodium in 5 ml suspension	VETERINA d.o.o., Croatia	Milk – 6 milkings Meat – 7 days
5	CEFAXIMIN® - L	100 mg rifaximin 200 mg cefacetril sodium in 5 ml suspension	Fatro S.p.A. – Ozzano Emilia (Bologna) Italy	Milk – 7 milkings Meat – 5 days
6	COBACTAN® LC	75 mg cefquinome sulfate in 8 g suspension	HOECHST, Germany	Milk – 7 milkings Meat – 2 days
7	KLAVUXIL®	200 mg amoxicillin trihydrate 50 mg clavulanic acid 10 mg prednisolone in 3 g suspension	Pliva, d.d., Croatia in cooperation with Pfizer	Milk – 4 milkings Meat – 7 days
8	KLOKSAFORT® LC	458 mg cloxacillin sodium 1.000.000 IU colistin sulfate in 10 ml suspension	KRKA tovarna zdravil d.d. Novo Mesto, Slovenia in cooperation with VIRBAC SA – 06516 – Carros, France	Milk – 10 milkings Meat – 3 days
9	KLOKSAVET® M	200 mg cloxacillin sodium 10 mg prednisolone acetate in 5 ml suspension	VETERINA d.o.o., Croatia	Milk – 6 milkings Meat – 7 days
10	LINCOCIN® FORTE S	330 mg lincomycin chloride 100 mg neomycin sulfate in 10 ml water for application	PHARMACIA, Animal Health Division, Belgium	Milk – 5 milkings Meat – 1 day
11	LINKOMICIN F	330 mg lincomycin 100 mg neomycin 1 mg dexamethasone-21-phosphate in 10 ml suspension	LEK, tovarna farmacevtskih in kemičnih izdelkov, d.d., Ljubljana, Slovenia	Milk – 10 milkings Meat – 2 days
12	MAMEXINE	200m g cephalexin 100.000 IU kanamycin sulphate in 10 g suspension	UNIVET Ltd. Ireland	Milk – 8 milkings Meat – 7 days

Table 7 continues

13	MASTI VEYXYM® FORTE	606 mg sodium benzylpenicillin 200 mg neomycin sulphate 4 mg chymotrypsin 4 mg trypsin 2 mg papain in 20 g suspension	VEYX-PHARMA GmbH, Germany/ Vetconsult Pharma d.o.o., Ljubljana	Milk – 12 days Meat – 7 days
14	MASTIJET FORT®	200 mg tetracycline chloride 250 mg neomycin sulfate 2.000 IU bacitracin 10 mg prednisolone in 8 g excipient (oil)	INTERVET International Boxmeer, Netherlands	Milk – 8 milkings Meat – 14 days
15	MASTIQUICK®	100 mg procaine bezylpenicillin 100 mg streptomycin sulfate 10 mg prednisolone excipient 5 g	Pliva d.o.o. Croatia	Milk – 6 milkings Meat – 7 days
16	ORBENIN® L.A.	200 mg cloxacillin excipient to 3 g	PFIZER Italiana S.p.A., Italy	Milk – 7 milkings Meat – 7 days
17	PATHOZONE®	250 mg cefoperazone sodium in 10 ml suspension	Pfizer Animal Health S.A. Italy	Milk – 7 milkings Meat – 2 days
18	PERACEF®	100 mg cefoperazone dihydrate in 10 ml suspension	PFIZER ANIMAL HEALTH, Latina, Italy	Milk – 10 milkings Meat – 2 days
19	RILEXINE 200 LC	200 mg cephalexin monohydrate in 10 ml suspension	KRKA, d.d. Novo Mesto, Slovenia in cooperation with VIRBAC, France	Milk – 2 milkigns Meat – 4 days
20	SYNULOX® LC	200 mg amoxicillin trihydrate 50 mg clavulanic acid 10 mg prednisolone in 3 g oily suspension	Pfizer Animal Health S.A. Italia	Milk – 4 milkings Meat – 7 days
21	TETRA DELTA®	100 mg novobiocin 150 mg neomycin 100.000 IU procaine benzylpenicillin 125 mg dihydrostreptomycin 10 mg prednisolone in 10 g oily suspension	PHARMACIA & UPJOHN, Puurs, Belgium	Milk – 6 milkings Meat – 7 days
22	UMBROCELAN FOAM JET	1.5 g procaine penicillin G 0.5 g neomycin sulfate in 13.7 g excipient	BOEHRINGER INGELHEIM-VETMEDICA, Germany	Milk – 10 milkings Meat – 6 days

Table 7 continues

23	VETRAMYCIN®	1.000.000 IU procaine benzylpenicillin 10.000 IU retinil palmitate 100 mg calcium d-pantothenate excipients: 1.8 mg butylated hydroxytoluene, 90 mg glycerol monostearate, 4.59 g liquid paraffin, in 9 g white vaseline	Alvetra und Wefft, Vienna, Austria	Milk – 8 milkings Meat – 5 days
24	XICLAV®	200 mg amoxicillin 50 mg clavulanic acid in 5 ml suspension	LEK, Ljubljana, Slovenia	Milk – 4 milkings Meat – 7 days

*Withhold periods may differ according to country.

Table 8. Intramammary preparations for treating bovine mastitis during dry period used in Slovenia (Uradni list RS, 13/2003; Uradni list RS, 17/2014).
Preglednica 8. Pripravki za intramamarno dajanje za zdravljenje mastitisa v času presušitve, ki so registrirani v Sloveniji (Uradni list RS, 13/2003; Uradni list RS, 17/2014).

No.	Drug	Composition	Manufacturer	Withhold period
1	ALBADRY® PLUS	400 mg novobiocin sodium 200 mg procaine penicillin G in 10 ml suspension	PHARMACIA & UPJOHN, Puurs, Belgium	Milk – 6 milkings Meat – 30 days
2	CEFATRON®	390 mg cephapirin benzathine in 5 ml suspension	Fatro S.p.A. – Ozzano Emilia (Bologna) Italy	Milk – 6 milkings Meat – 42 days
3	CEPRAVIN® DRY COW	250 mg cefalonium dihydrate in 3 g suspension	Schering-Plough A.H. Uxbridge, Great Britain	Milk – 55 days Meat – 21 days
4	KLOKSAFORT® DC	874 mg cloxacillin benzathine in 9 g suspension	KRKA tovarna zdravil d.d. Novo Mesto, Slovenia	Milk – 10 milkings (if administered >35 days before calving) Meat – 28 days
5	MASTIDRY®	600 mg cloxacillin benzathine 300 mg ampicillin trihydrate	Veterina, d.o.o. Croatioa	Milk – 8 milkings (administered >49 days) Meat – 28 days
6	ORBENIN® EXTRA DRY COW	600 mg cloxacillin benzathine excipient to 3.6 g	Pfizer Animal Health S.A. Italia	Milk – 4 days after calving Meat – 28 days
7	ORBENIN® D.C.	500 mg cloxacillin excipient to 3 g	PFIZER Italiana S.p.A.m Italy	Milk – 4 days after calving Meat – 28 days
8	RILEXINE 500 DC	375 mg cephalexin benzathine in 8 g suspension	KRKA, d.d. Novo Mesto, Slovenia in cooperation with VIRBAC, France	Milk – 10 milkings if dry period lasts < 60 days Meat – 0 days
9	SICCOVET®	1.000.000 IU procaine benzylpenicillin 1.000.000 IU dihydrostreptomicin sulphate 10.000 IU retinil palmitate 100 mg potassium d-pantothenate excipients: 1.8 mg butylated hydroxytoluene, 306 mg aluminium monostearate, 1.800 mg white vaseline in 9.000 mg liquid paraffin	Alvetra und Wefft, Vienna, Austria	Milk – 5 days after beginning of lactation Meat – 21 days
10	SUPER MASTIDOL DC	500.000 IU penicillin G potassium 1.000.000 IU procaine penicillin G 500 mg neomycin sulfate excipient to 9 g	INTERVET International, Netherlands	Milk – 5 weeks Meat – 10 weeks

2.9 DETERMINATION OF MAXIMUM RESIDUE LIMITS (MRL)

Determination of permitted maximum residue limits in food, which can be ingest, is based on biological, pharmacological and toxicological studies. Before determining the MRL, it is necessary to determine Acceptable Daily Intake (ADI), which is the daily intake of a particular substance over a lifetime without having any adverse effects on human health. ADI is expressed on a body weight basis and is calculated from the results acquired from pharmacological, toxicological and microbiological studies. ADI is usually based on no observed (adverse) effect level (NO(A)EL) or sometimes on lowest observed (adverse) effect level (LO(A)EL) in the most sensitive appropriate test species. In the assessment of ADI an uncertainty factor – safety factor (SF) of 100 is used; and in certain cases of 1000. Uncertainty factor is used due to the extrapolation from experimental studies in animals to humans, assuming that humans are 10 times more sensitive than animals and because of the differences within population. Thus, ADI (µg/kg) is NO(A)EL x 0,01 SF. When ADI is established, MRL can be determined for the individual food commodities concerned. When establishing MRL for milk, the effects on dairy starter cultures must be considered (Volume 8, 2005).

It should be noted that there is no simple equation for determination of MRL. The establishment of MRL value, in addition to ADI value, include few other key points:

- The basis for the calculation: arbitrary body weight of the consumer (60 kg x ADI (µg/kg)) and consumption figures (Food basket);
- Marker residue (parent drug or its metabolites or combination of any of these) deducted from the results of the depletion studies in edible tissues, as well as ratio of the marker residue concerning the total residues;
- Tissue distribution;
- MRLs and calculation of the amount of residues likely to be ingested.

The edible tissues for mammals are muscle, liver, kidney and fat or fat together with skin in pigs and poultry. For fish muscle and skin in natural proportions (Volume 8, 2005). When

proposing MRL, ADI must not be surpassed after considering intake from all sources shown in the food basket (Table 9).

Table 9. Daily food basket of food of animal origin (Volumen 8, 2005).
Preglednica 9. Dnevna prehrambena košarica živil živalskega izvora (Volumen 8, 2005)

Mammals		**Poultry**		**Fish**		**Bees**	
Muscle	300 g	Muscle	300 g	Muscle	300 g(2)	Honey	20 g
Fat	50 g(1)	Fat	90 g(1)				
Liver	100 g	Liver	100 g				
Kidney	50 g	Kidney	10 g				
Milk	1500 g	Eggs	100 g				

(1) Fat and skin in natural proportions (only in pigs for mammals)
(2) Muscle and skin in natural proportions

2.10 DETERMINATION OF WITHHOLD PERIOD (WP)

For granting marketing authorisation of a veterinary medicinal product for food producing animals, the WP must be determined. The WP is the interval after the last administration of the veterinary medicinal product, under normal conditions of use, during which the animal must not be slaughter or its products (milk or eggs) must not be used for human consumption, ensuring that the residues are below the MRL. The withholding period for milk is calculated in milkings, in animals milked twice a day, because the predominant milking scheme is twice a day.

Different methods are available and used to establish WPs in milk. According to the European Medicinal Agency the harmonized method in EU for determining WP is the "Time to Safe Concentration" (TTSC). It determines the time necessary for the measured concentration to drop below the MRL, and stay below the MRL at later times. The residues are determined individually; healthy animals are given the product containing the drug in question and are milked twice a day. At least 19 healthy animals are included in the study to ensure 95% confidence level. A representative sample from the relevant population include high yielding cows in early stage of

lactation, and low yielding cows in late stage of lactation. The WP is evaluated by the manufacturer of the drug preparation (EMEA, 2000).

2.11 SCREENING ANTIMICROBIAL RESIDUES IN MILK

The first microbiological tests for antimicrobial residue detection in milk were developed in the 1940s (Bishop & White, 1984). Since then many new tests, including enzymatic, immunological and receptor-binding assays, have been developed and their performances have been improved. Nowadays, in EU each screening test that meets the criteria posed in the Commission Decision 2002/657/EC is eligible for use in analytical purposes.

Screening methods are designed to detect the presence of a substance or class of substances at the level of interest, meanwhile to avoid false compliant results. They should be relatively inexpensive and capable of analysing large amount of samples in reasonable time.

Microbial inhibitor tests are widely used for antimicrobial screening in milk. They mainly target β-lactam drugs. Delvotest-P was firstly introduced in 1975 by Gist-Brocades Laboratories in the Netherlands (Bishop & White, 1984). During the past decades the performances of Delvotest® were improved and currently it is one of the most widely used in the dairy industry. Other widely used screening tests include Twinsensor^{BT} receptor binding test for penicillins, cephalosporins and tetracyclines, Charm– radioimmunoassay test for β-lactams, SNAP tests, etc.

The screening methods used must fulfill the requirements stated in the Commission Decision 2002/657/EC. Each screening method that does not give false negative results greater than 5% at the level of interest (MRL) can be used for screening purposes. However, eventually all positive results must be confirmed by a confirmatory method (2002/657/EC).

Chemical methods unlike microbial methods are generally too specific and more expensive to be applied as an initial screening. On the other hand microbial methods are cheaper but in order to detect wider spectrum of antimicrobials, additional bacterial strains should be used thus requiring more time and effort (Pikkemaat, 2009).

However, a screening test does not allow a quantitative determination of the residue content. For quantification of the residue content a physico-chemical confirmatory test must be performed. If a confirmatory test is not performed, milk with a positive screening test result is considered as being insecure (Heeschen & Suhren, 1996).

Milk with a residue concentration above the MRL is not allowed for human consumption and must not be processed nor diluted to a concentration below the limit.

2.12 REGULATION

Each Member State in the EU has established national monitoring plan for veterinary drug residue in live animals and animal products in accordance with the requirements laid down in Council Directive 96/23/EC (EC, 1996), Commission Decision 97/747/EC (EC, 1997), 98/179/EC (EC, 1998) and the Regulation 2017/625 (EC, 2017). The aim of the monitoring is to ensure that no illegal treatments have been used, the MRLs for veterinary drug residues have not been exceeded and to uncover the reasons that caused the residues to appear in the food of animal origin (EC, 1998). Only antimicrobials with determined MRL in milk are eligible for use in dairy cows (2010/37/EC). When a positive sample exceeds the MRL, the competent authority should as soon as possible identify the animal/s and farm of origin or departure. Thereof, to recognize the reasons that contributed the presence of residues and prevent the animals or products intended for human consumption reach the market (EC, 1996).

Bulk milk tanks occasionally test positive to antimicrobials, nevertheless their frequency, unlike in the past, is highly reduced. The problem with slow and prolonged excretion of antimicrobials in milk from treated mastitic cows that exceeds the MRL has been reported in a few articles (McEwen et al., 1991, 1992; FDA, 2015) but has not received much attention.

3. MATERIALS AND METHODS

3.1 SAMPLING

The study included 97 Holstein Friesian cows with clinical mastitis from three commercial dairy farms (with 150 to 200 dairy cows). Cows had access to drinking water *ad libitum* and were fed twice daily after milking with a total mix ration (based on home produced forages: grass silage, maize silage, hay and straw; concentrates and vitamin-mineral supplement) according to National Research Council (2001) recommendations. The dairy farms had a free-stall housing system. On all three farms cows were milked twice daily, in a standard milking parlour, and were producing an average 8800 kg and 9000 kg liquid milk per lactation respectively. Each farm had daily veterinary attendance.

Cows enrolled in the study had no concomitant diseases diagnosed. The veterinarians chose all treatments for the mastitis. All cows were treated by intramammary route with antimicrobial drugs and most of them by the parenteral route. The attending veterinarians completed a questionnaire (Appendix A), providing data about the cow; prescribed WP, age, lactation number, number of infected udder quarters, basic clinical signs (fever, quarter oedema, decreased milk production, abnormal milk) and treatments applied.

We collected milk samples from infected quarters in 66 cows for bacterial identification before the treatment. Milk samples were collected aseptically in sterile 10 ml test tubes and kept at 4-8 °C until bacteriological analysis. Identification of the bacteria responsible for the infection was carried out within 24 h.

Post treatment milk samples for drug residue analyses were taken from the mastitic quarters and from untreated healthy quarters in the same cow. Milk samples were collected at two milkings before, and two milkings after the prescribed WP. In 20 cows (Cows 36-48, 61-68), due to extended excretion we sampled one milking before the prescribed WP and 4 milkings after the WP. From 48 cows (Cows 1-35; 49-60) we sampled milk also from the untreated healthy quarters to check if crossover of drugs had occurred. The milk samples were collected from totally 100 treated quarters from 97 lactating cows presenting clinical mastitis. For this purpose 25 ml containers were used and they were stored at -20 °C until analysis. Altogether, we collected 677 milk samples for antibiotic analysis and pathogen detection.

3.2 ANALYTICAL PROTOCOL

Before the beginning of the treatment we collected 66 milk samples from the infected quarter/s for detection of the infectious agent. Mastitis-causing pathogens were identified by Matrix-Assisted Laser Desorption Ionization-Time of Flight mass spectrometry (MALDI-TOF, Bruker Daltonics, Bremen, Germany).

All samples taken from the healthy untreated mammary quarters before and after the prescribed WP were analysed by a microbiological method using five-plates, so-called ‘the STAR protocol’ (Gaudin et al., 2004).

In order to cover all antimicrobials used in the treatment, the samples were analysed by the STAR protocol, Delvotest® SP-NT (DSM, Delft, the Netherlands) and Twinsensor^{BT} KIT020 (Unisensor, S.A., Angleur, Belgium). Firstly, we analysed the milk samples collected after the prescribed WP. If these samples were negative on antimicrobials, the samples collected before the prescribed WP were not analysed. If a positive sample was detected, all milk samples taken from that quarter, were analysed by the appropriate quantitative method (quinolones with HPLC-fluorescence detector, tetracyclines with LC-MS/MS, and penicillins with UPLC-MS/MS). The protocol regarding drug residue analysis is schematically presented in Figure 3. The performances of screening tests and confirmative methods are presented in Table 10.

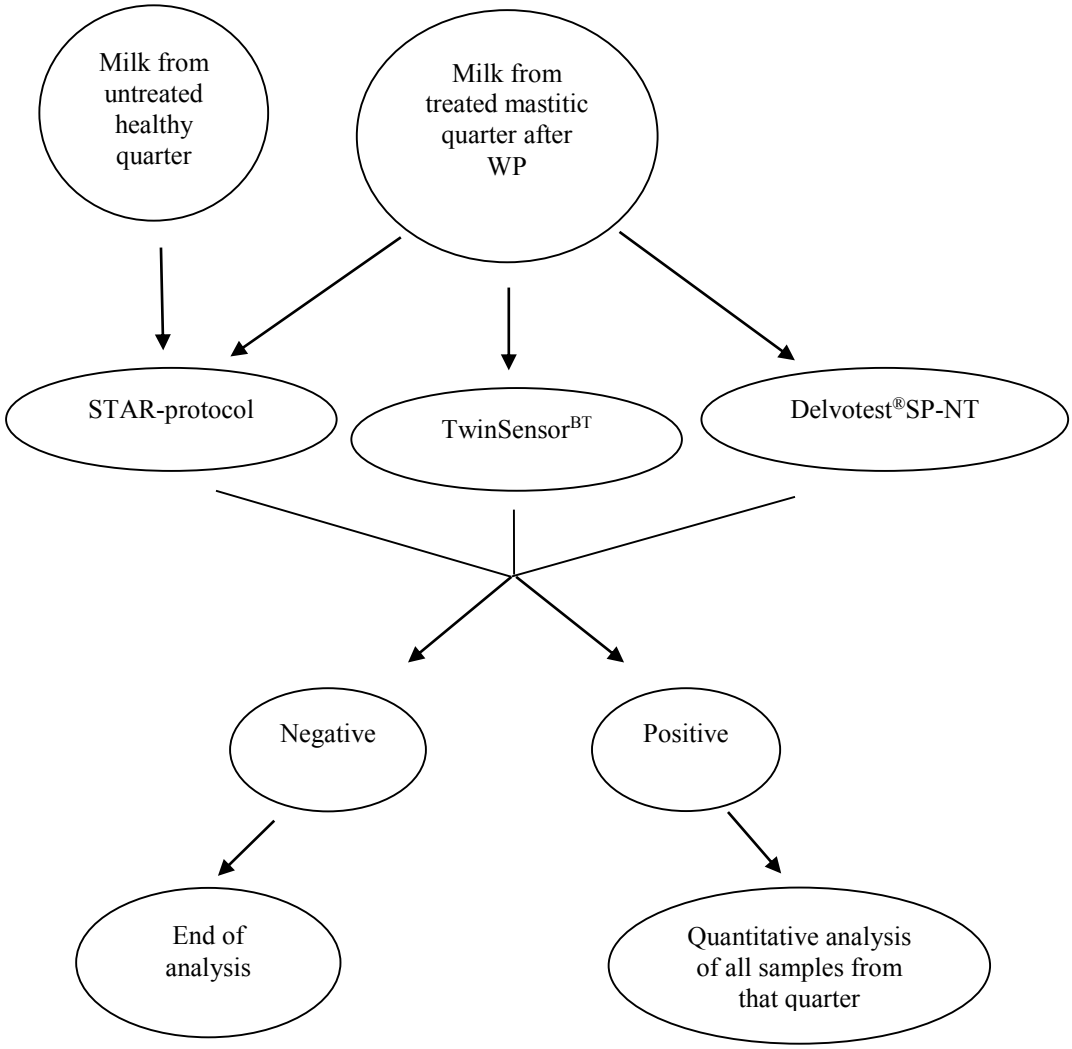


Figure 3. A schematic diagram showing the examination of milk for the presence of antimicrobials. **Slika 3.** Šematski prikaz poteka preiskav mleka na prisotnost protimikrobnih učinkovin.

3.3 ANALITICAL METHODS

3.3.1 Bacterial identification

Each milk sample was plated onto blood agar (Trypticase soy agar with 5% sheep blood) and incubated at 37 °C for 18 to 24 h. Mastitis-causing pathogens were identified by Matrix-Assisted Laser Desorption Ionization-Time of Flight mass spectrometry (MALDI-TOD, Bruker

Daltonics, Bremen, Germany). A fresh colony was smeared onto a ground steel MALDI target plate in a thin film and covered with 1,5 µl of a saturated solution of α-cyano-4-hydroxy-cinnamic acid in 50% acetonitrile-2,5% trifluoroacetic acid. The mass spectrum was evaluated in the linear positive mode (Microflex; Bruker Daltonics, Bremen, Germany). The mass spectra (ranging from 2.000 to 20.000 Da) were compared with the reference spectra of the integrated database provided by the manufacturer, using MALDI Biotyper software (Bruker Daltonics, Bremen, Germany).

3.3.2 Antimicrobial analysis

Standards and standard solutions: Amoxicillin trihydrate, penicillin G potassium salt, oxacillin sodium salt monohydrate, cefquinome sulfate, cephalexin, tetracycline hydrochloride, neomycin trisulfate salt hydrate, gentamicin sulfate salt, kanamycin A disulfate salt hydrate, dihydrostreptomycin sesquisulfate, streptomycin sesquisulfate hydrate, novobiocin sodium salt, marbofloxacin, enrofloxacin and ciprofloxacin were sourced from Sigma-Aldrich (St. Louis, MO, USA).

3.3.3 Screening Tests

3.3.3.1 TwinSensor^{BT} Kit020

The TwinSensor^{BT} Kit020 (Unisensor, S.A., Angleur, Belgium) is a competitive test containing two receptors for the detection of tetracyclines and β lactam antibiotics. Sample analysis and validation were carried out as described in the manufacturer's manual and in the article of Perme et al. (2010), respectively. The sensitivity limits for amoxicillin, penicillin, cefquinome, cefalexin and tetracycline are listed in Table 10. Two hundred microliters of thawed milk sample was transferred into the microwell containing receptors and antibodies linked to gold particles, then mixed and incubated (Heatsensor, Aerne Analytic, Hamburg, Germany) for 3 min at 40 °C. A dipstick, composed of a set of membranes on captured lines, was immersed into the microwell and incubated for another 3 min. The dipsticks were read visually and by ReadSensor (Unisensor, S.A., Germany). In each analysis, one positive and one negative control were added.

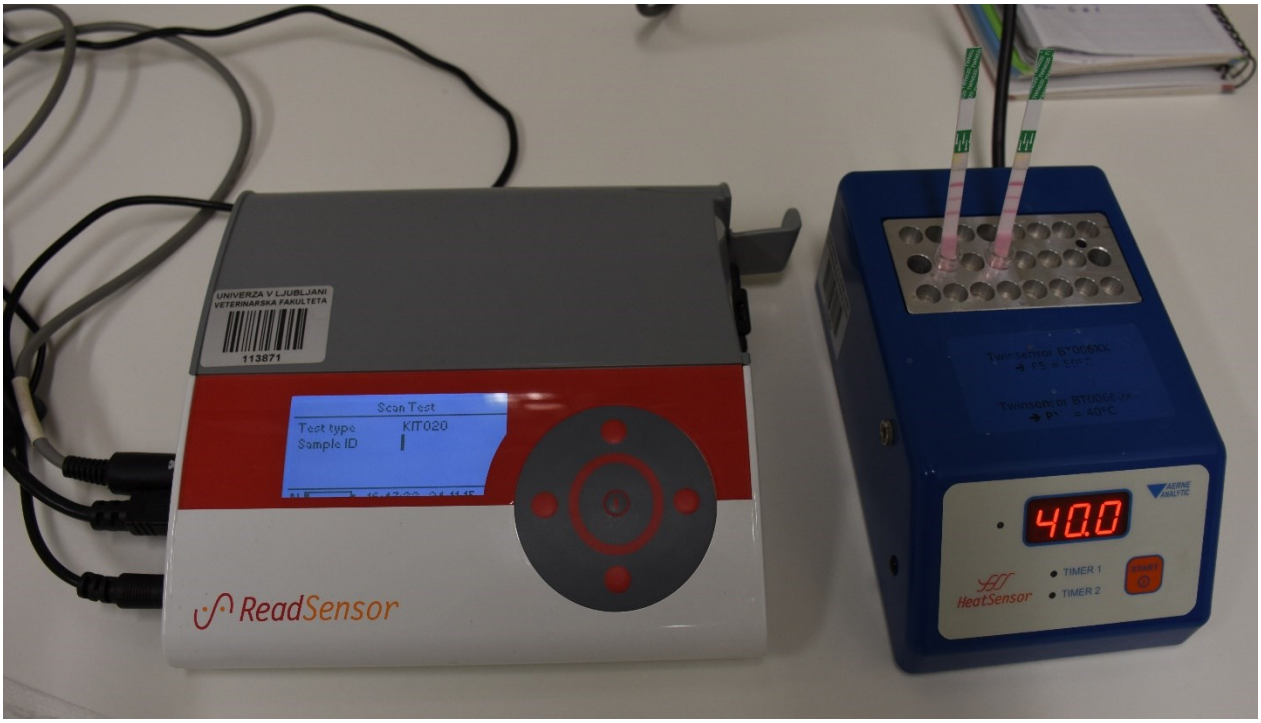


Figure 4. Heatsensor (right) with two dipsticks and Readsensor (left). From the picture it can be noticed that both dipsticks are negative on penicillins and cephalosporins.
Slika 4. Heatsensor (desno) z dvema merilnima lističema in Readsensorjem (levo). Iz slike lahko opazimo, da sta oba merilna lističa negativni na peniciline in cefalosporine.

3.3.3.2 Delvotest® SP-NT

The Delvotest® SP-NT (DSM Food Specialties, Delft, The Netherlands) is a microbial inhibitor test consisting of agar ampoules that contain a solid and buffered agar medium with all necessary nutrients, a standardized number of spores of the test organism *Bacillus stearothermophilus var. calidolactis*, and a pH indicator (bromocresol purple). The method was validated according to the European Commission Decision 2002/657/EC. Milk samples were analysed according to the manufacturer's instructions. Before analysis milk samples were preheated at 80 °C for 5 minutes. One hundred microliters of milk was transferred into the test ampoule and incubated at 64 ± 0.5 °C for 3 hours. A purple colour of the ampoule indicated a positive sample, and a yellowish colour indicated a negative sample. In each analysis run, positive and negative controls were included. The sensitivity limits for Delvotest® SP-NT are listed in Table 10.

3.3.3.3 STAR protocol; a five-plate microbiological test for antimicrobial detection

The STAR protocol is a five-plate microbiological test for detection of antimicrobial residues in milk, meat, feed, and eggs, was used with:

- *Bacillus cereus* ATCC 11778 (KWIK-STIK, Microbiologics, MN, USA) for tetracyclines,
- *Escherichia coli* ATCC 10536 (KWIK-STIK, Microbiologics, MN, USA) for quinolones,
- *Staphylococcus epidermidis* ATCC 12228 (KWIK-STIK, Microbiologics, MN, USA) for aminoglycosides,
- *Bacillus subtilis* B.G.A. spore suspension (Merck, Darmstadt, Germany) for aminoglycosides and rifamixin,
- *Kocuria rhizophila* ATCC 9341 (KWIK-STIK, Microbiologics, MN, USA) for penicillines and cephalosporines.

The test is based on the inhibition of growth of the test microorganisms around the sample in presence of antimicrobials (Gaudin et al., 2004). The KWIK-STIK package was supplied with a fluid for bacterial rehydration. The manufacturer's instructions were followed for preparation of the bacterial strains. The rehydrated bacteria were inoculated onto blood agar (BA) and slant TSA (Tryptic Soy Agar) and incubated for 24 hours at 37 ºC. After the incubation, the BA plates were wrapped with plastic foil and stored in a refrigerator at 4 – 8ºC for up to two months (*B. cereus* was stored for one month only) for further subculturing. The purity of the culture was assessed visually and when necessary the colonies were stained by Gram's method and assessed with a light microscope. The colonies grown on the slant agar were used for preparing bacterial suspension.

For media preparation the following three culture media were used: Antibiotic (AT) agar No. 1 at pH 8 (Merck, Darmstadt, Germany) and Antibiotic agar No. 5 at pH 6 and 8 (Merck, Darmstadt, Germany). The agars were prepared according to the manufacturer's instructions. The pH values 6 and 8 were adjusted by adding appropriate amount of 37% HCl and NaOH, respectively. After autoclaving (121ºC, 15 min), the pH values were adjusted once again. The density of the bacterial suspension in saline solution was adjusted using a spectrophotometer (Varian Cary 50 Probe, Santa Clara, CA, USA). Five millilitres of the inoculated medium was applied on Ø90 mm Petri dishes

and left to cool on a cold horizontal surface. In each analysis run, positive and negative controls were added. Details of the preparation are listed in Table 11.

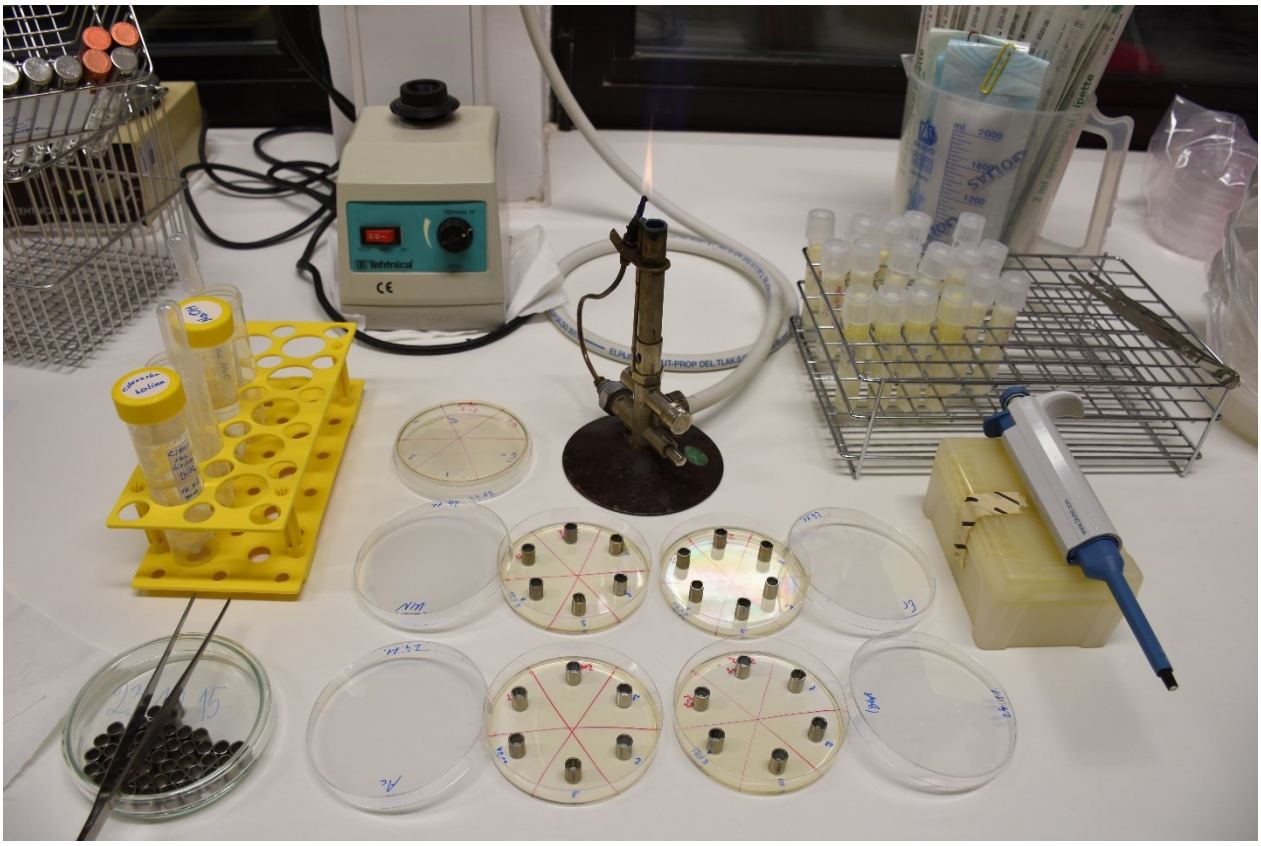
Table 10. Performances of screening and confirmatory tests for different antimicrobials compared to their maximum residue limit in milk.
Preglednica 10. Izvedba presejalnih in potrditvenih testov za različne protimikrobne snovi glede na najvišje mejne vrednostmi ostankov v mleku.

Antimicrobials	MRL[1] in milk	LOD screening			Confirmatory	
		Twinsensor[2]	Delvotest® SP-NT[3]	STAR protocol[4]	LOQ[5]	CC□[6]
Amoxicillin	4	3-4	2-3	8	2	5.0
Penicillin	4	2-3	2	4-6	2	4.3
Cefquinome	20	20-30	65-75	40	/	/
Cephalexin	100	>750	45	100	/	/
Tetracycline	100	80-100	270-320	100	5	110
Oxytetracycline	100	50-60	250-300	100	5	/
Neomycin	1500	NS	115-190	50	/	/
Gentamycin	100	NS	50	50	/	/
Kanamycin	150	NS	5000	300	/	/
Dihydrostreptomycin	200	NS	700	150	/	/
Novobiocin	50	NS	750-800		25	56.0
Marbofloxacin	75	NS	>1000	30	25	/
Enrofloxacin & Ciprofloxacin	100	NS	1000-1500	100	5	65.5
		NS	/	/	20	55.8

[1] Maximum residue limit: Commission Regulation No 37/2010 (EC, 2010) in µg/kg.
[2] Limit of detection stated in the test instruction Twinsensor^{BT} KIT020 in µg L^{-1}.
[3] Limit of detection stated in Technical Bulletin Delvotest SP-NT (Delvotest SP-NT, 2012) in µg L^{-1}.
[4] Limit of detection obtained by in-house validation in the Institute of Food Safety, Feed and Environment.
[5] Limit of quantification obtained by in-house validation in the Institute of Food Safety, Feed and Environment.
[6] Detection capability obtained by in-house validation in the Institute of Food Safety, Feed and Environment.
NS – Not Sensitive.

Before analyzing, milk samples were preheated at 80 °C for 5 minutes. For tetracycline, the samples were prepared as described by Raspor Lainšček *et al.* (2014). One hundred microliters of sample was applied onto each of test plates in a stainless steel cylinder (Ø8 ± 0.2 mm, height 10 ± 0.2 mm) and incubated as shown in Table 11. When the diameter of the zone of inhibition was greater than 8.1 mm on at least one of the five plates, the sample was considered positive. In each analysis run, positive and negative controls were added.

Figure 5. Five plate test – STAR protocol. The samples are prepared for inoculation on the agar plates.
Slika 5. Test s petimi ploščami – protocol STAR. Vzorci mleka so pripravljeni za inokulacijo na agar plošče.



Neomycin concentration was estimated from a calibration curve from the plate with *Staphylococcus epidermidis,* and cefalexin and cefquinome from the plate with *Kocuria rhizophila*. The concentration of neomycin, cefalexin and cefquinome in milk showed a good correlation with the size of the inhibitory zones, thus the concentrations in milk were estimated from the calibration curves.

Table 11. STAR protocol − a five-plate microbiological test: Bacterial strains and its suspension absorbance used for the plate preparation, specific preparation protocol for each antibiotic plate, antibiotic group detection for each plate and incubation temperatures that need to be used for each plate.
Preglednica 11. Protokol STAR - mikrobiološki test s petimi ploščami: bakterijski sevi in njihova absorpcija suspenzije, ki se uporabljajo za pripravo plošče, specifični protokol priprave za vsako antibiotično ploščo, detekcija antibiotične skupine za vsako ploščo in inkubacijske temperature, ki jih je treba uporabiti za posamezno ploščo.

Bacteria	*Bacillus cereus* (E – plates)	*Kocuria rhizophila* (Ac - plates)	*Staphylococcus epidermidis* (Er - plates)	*E. coli* (Kin - plates)	*Bacillus subtilis* (Ampule)
Detection of antibiotic group	Tetracyclines	β-lactams, Cephalosporins, Macrolides	Aminoglycosides, Novobiocin, Rifaximin	Quinolones, Bacitracin	Aminoglycosides
Bacterial suspension absorbance	1.0 – 1.1	0.5 – 0.60	0.25 – 0.30	0.30 – 0.35	Ampule
cfu mL^{-1} in agar	10^5-10^6	1-9 x 10^4	5 x 10^4	3 x 10^5	3 x 10^5
Preparation of antibiotic plates	500 µL BS AT5 pH 6 200 µl CHP	300 µL BS AT1 pH 8	100 µL BS AT1 pH 8	150 µL BS AT5 pH 8	200 µL BS AT5 pH 8
Incubation (16-20 h)	30 °C	30 °C	37 °C	37 °C	30 °C
Positive control	tetracycline	oxacillin	neomycin	enrofloxacin	streptomycin & kanamycin

AT – Antibiotic agar.
BS – Bacterial Suspension.
CHP – Chloramphenicol 500 µg mL^{-1}.

Preparation of bacterial suspension and test plates

Slant TSA tubes were used for preparation of bacterial suspension. After incubation of the bacterial strains at 37ºC for 24 hours the agar was left to cool at room temperature (20 - 25ºC). Suspension was made by adding saline solution. The suspension density was adjusted by a spectrophotometer at 380 nm by adding saline solution.

The antibiotic agars were prepared according to instructions' label, autoclaved and were left in a water bath at 45ºC. The bacterial suspension was mixed with 50 ml culture media and applied on Ø9 cm Petri dishes. On each Petri dish was applied 5 ml of the agar and left to cool on a cold surface. The test plates were stored in a refrigerator (4 - 8ºC) and used in the next 7 days. Test plate **E** (*B. cereus*) was used within 2 days from the preparation.

Preparation of *Bacillus cereus* suspension and E - plates

Two and a half millilitres of sterile saline solution were added in the slant TSA tube with *B. cereus*. The culture was scraped with a sterile bacteriological loop. Point three millilitres was pipetted into an empty sterile tube and mixed with Vortex for 15 seconds. When homogenous suspension was obtained, 9 ml of a saline solution was added. The desired density (1.0 – 1.1) was assessed by a spectrophotometer.

Fifty millilitres of AT5 pH6 agar at 45-50ºC were mixed in 100 ml Erlenmeyer flask together with 0.5 ml suspension of *B. cereus* and 200 µl (concentration 500 µl L^{-1}) of chloramphenicol (Sigma - Aldrich). Thereafter, 5 ml of the prepared agar was pipetted into Ø9 mm Petri dishes. Desired concentration of *B. cereus* in the agar was 10^5 to 10^6 cfu/ml.

Preparation of *Kozuria rhizophila* suspension and Ac – plates

Two and a half millilitres of sterile saline solution was added in the slant TSA tube and the culture was scraped with a sterile bacteriological loop. Point fifteen millilitres was than pipetted in a tube containing 9 ml saline solution and mixed by Vortex. The desired density was 0,55 – 0,6.

Fifty millilitres of AT5 agar pH8 at 45-50 ºC were mixed in 100 ml Erlenmeyer flask together with 0,3 ml suspension of *K. rhizophila*. The agar was then pipetted in Petri dishes, each plate containing 5 ml of the agar suspension. The desired concentration of *K. rhizophila* was 1-9 x 10^4 cfu/ml.

Preparation of I_{BGA} – plates

Ready-to-use *B. subtilis* suspension was purchased by Merck (Darmstadt, Germany) with concentration of 3 x 10^5 cfu/ml. Point two millilitres of *B. subitils* suspension were added into 50 ml AT5 pH8 agar and mixed. Five millilitres of the agar was then pipetted in Petri dishes. The plates were left to cool on a cold surface before being used.

Preparation of *Staphylococcus epidermidis* suspension and Er – plates

Two and a half millilitres of sterile saline solution was added in the slant TSA tube and the culture was scraped with a sterile bacteriological loop. Point one millilitre of the suspension was pipetted into tube with 9 ml saline solution and mixed. The desired density assessed by a spectrometer was 0.25 – 0.3.

Fifty millilitres of AT1 pH 8 agar, at 45-50 ºC temperature were mixed in Erlenmeyer flask together with 0,1 ml *S. epidermidis* suspension. The agar was pipetted in Petri dishes, each plate containing 5 ml. The desired concentration of *S. epidermidis* was 5 x 10^4 cfu/ml.

Preparation of *E. coli* suspension and KIN – test plates

Two and a half millilitres of sterile saline solution was added in the slant TSA tube. The culture was scraped with a sterile bacteriological loop. Point one millilitre of the suspension was pipetted into tube with 9 ml saline solution and mixed. The desired density assessed by a spectrometer was 0.3 – 0.35.

Fifty millilitres of AT5 pH8 at 45-50ºC were mixed in Erlenmeyer flask together with 0.15 ml of *E. coli* suspension. In each Petri dish 5 ml of the agar was pipetted and left to cool. The desired concentration of *E. coli* was 3 x 10^5 cfu/ml.

Preparation of standard solutions and milk samples

To test the sensitivity of the test plates, positive controls with known antibiotic concentrations were applied on each analysis run. The antibiotic standards provided by Sigma-Aldrich were dissolved in appropriate solvents (to make primary standard solutions) as follows: tetracycline in phosphate buffer (Merck, Darmstadt, Germany) with pH value 4,5, oxacillin in phosphate buffer with pH 6, streptomycin, neomycin and enrofloxacin in phosphate buffer with pH 8. From these primary standard solutions positive controls were made. Raw milk free of antimicrobials was used for preparation of positive controls in the following concentrations: 40 µg/L tetracycline for E – plate, 100 µg/L oxacillin for Ac – plate, 150 µg/L streptomycin for I_{BGA} – plate, 300 µg/L neomycin

for Er – plate and 30 µg/L enrofloxacin for KIN – plate. The standard solutions were stored in a freezer at -20ºC until use.

The milk samples were mixed before 10 ml were transferred into 15 ml tubes. Each tube was then heated at 80ºC for 5 minutes. For detection of tetracycline residues (E – plate) a special preparation of the sample was needed. In 10 ml of the sample 4 drops (200µl) of citric acid (1g/10 ml distilled water) was added and mixed. One hundred microliters of the sample was applied on each test plate in a stainless steel cylinder (Ø8 ± 0.2 mm, height 10 ± 0.2 mm) and incubated. On each test plate maximum of 6 test samples were applied. The test agar plates were incubated for 16 – 20 hours; plates E, Ac, and I_{BGA} at 30 ± 1ºC, plates Er and KIN at 37 ± 1ºC. The results were read after the incubation period. Zones around the cylinder were measured by caliper gauge. Zones wide ≥ 8.1 mm were considered positive.

Evaluation of results

The results of the 5-Plate method can be evaluated both qualitatively and quantitatively. Qualitative results are obtained by analyzing the effect of antimicrobials on a combination of sensitive and resistant bacterial strains. If the sample contains a known antimicrobial, its concentration can be assessed quantitatively. When a sample was positive on cefquinome, cephalexin, dihydrostreptomycin or neomycin a calibration curve was created for quantitative analysis. For this purpose preheated raw milk at 80°C for 5 minutes was used. Their concentrations in milk showed a good correlation with the size of the inhibitory zones, hence they were estimated from the calibration curves.

3.3.4 CONFIRMATORY METHODS

3.3.4.1 Enrofloxacin and ciprofloxacin analysis with HPLC-fluorescence detector

We followed the procedure of Kirbiš et al. (2005) with minor modifications. Briefly, after extraction of quinolones into acetonitrile (MeCN), the organic phase was evaporated under a

nitrogen stream, and the water phase defatted with *n*-hexane. Chromatography was carried out using an HPLC, consisting of a quaternary pump, a vacuum degasser, a temperature controlled automatic injector and a column thermostat connected to fluorescence detector (Agilent Technologies 1100 series, San Diego, CA, USA). The separation was achieved on a PLRP-S (150 mm x 4.6 mm, 3 μm) analytical column in combination with a PLRP-S (5 x 3 mm, 5 μm) guard cartridge (Polymer Laboratories, Shropshire, UK). The injection volume was 30 μL, the flow rate 0.8 mL min^{-1}, and the column temperature 50 °C. Gradient chromatography was performed using phase A (85% 0.02 mol L^{-1} H_2PO_4 and 15% MeCN) and phase B (MeCN) in the following manner: 0 to 15.9 min 0% B, 16 to 28 min increasing to 10% phase B. Fluorescence was excited at 280 nm and emission read at 450 nm. The system was controlled and data processed using integration software ChemStation (Agilent Technologies). The method was validated according to the European Commission Decision 2002/657/EC (EC, 2002).

3.3.4.2 Tetracycline analysis with LC-MS/MS

Milk samples were prepared following the method of MacNeil *et al.* (1996) with minor changes, and measurements made according to Založnik *et al.* (2007). After deproteinisation of the samples with Carrez I and II, McIlvaine-EDTA buffer was added. After centrifugation the supernatant was purified on a Strata X 33 μm Polymeric reversed phase 200 mg, 6 mL (Phenomenex, Torrance, CA, USA) column. The evaporated methanol eluate was redissolved in mobile phase. Liquid chromatography was carried out on a Waters Alliance 2695 (Waters, Milford, MA, USA) chromatograph using a Gemini C18 3μ column (110A, 150 x 4.6 mm, Phenomenex) with a guard column SecurityGuard Cartrige Gemini C18 (4 x 3.0 mm ID, Phenomenex). The mobile phase was used in gradient mode and composed of phase A (0.1% formic acid) and phase B (MeCN). The gradient ran for 2 minutes with 2% B, which was increased to 95% B in 10 minutes, and maintained for 2 minutes. Flow rate was 0.4 mL min^{-1} and injection volume 10 μL. Column temperature was 25.0 °C. Mass detection was performed using a triple-quadrupole tandem mass spectrometer Waters Micromass Quatro Micro (Waters, Milford, MA, USA) with electrospray ionisation source operating in positive mode. The source block temperature was 120 °C and

desolvation temperature 350 °C. All data were processed using TargetLynx (Waters, Milford, MA, USA). For identification and quantification of tetracycline, m/z ions 445 > 410 and 445 > 154 were monitored. The method was validated according to the European Commission Decision 2002/657/EC (EC, 2002).

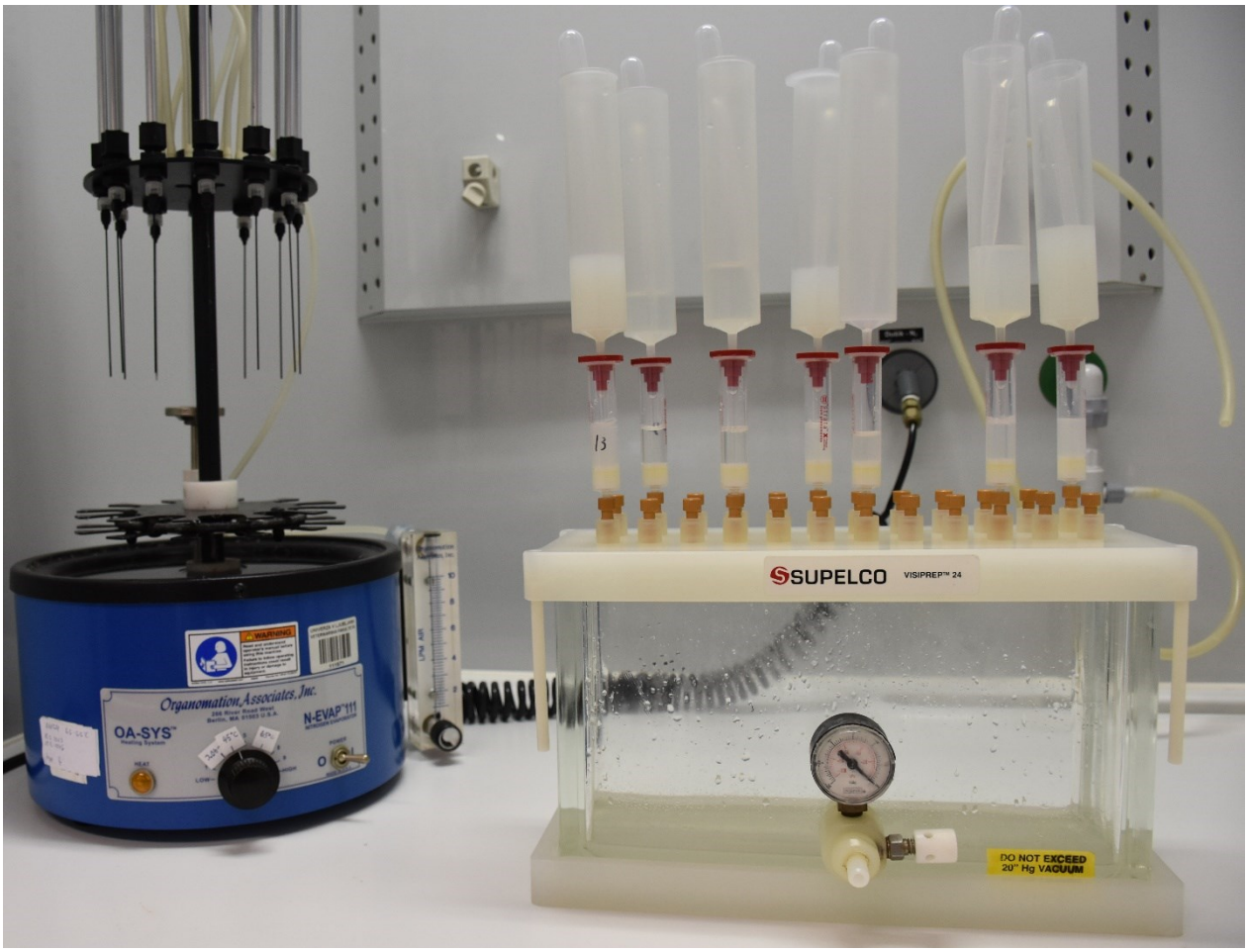


Figure 6. Tetracycline extraction - SPE cartridges with C18 packing positioned on the Vacuum manifold.
Slika 6. Ekstrakcija tetraciklina – SPE kolonice s C18 nosilcem so postavljene na Vacuum manifold.

3.3.4.3 Antimicrobial analysis with UPLC-MS/MS

Amoxicillin, procaine benzylpenicillin and novobiocin were identified, and quantified following the method of Pezdir *et al.* (2012), with minor changes. Milk sample was deproteinised with MeCN; after centrifugation the supernatant was filtered using Captiva filter (Captiva Non

DripLipid and Protein DetPlt, Agilent Technologies, USA). Measurement was carried out using a Waters UPLC® system connected to a Xevo-TQMS detector (Waters, Milford, MA, USA). The analytical column used was a Zorbax RRHD Eclipse Plus C8 (2.1 x 100 mm, 1.8 µm, Agilent Technologies). A gradient was applied with phase A (0.05% formic acid and 5% MeCN in water) and phase B (0.05% formic acid in MeCN). Gradient started with 0% B for 1.2 minutes, then increased to 65% in 5.8 minutes. Flow rate was 0.5 mL min^{-1}, column temperature 30.0 °C and injection volume 10 µL. The spectrometer operated in positive electrospray ionisation mode. Source block temperature was 150 °C and desolvation temperature 500 °C. All data were processed using TargetLynx (Waters, Milford, MA, USA). For identification and quantification m/z ions were for amoxicillin 366 > 114 and 366 > 208, for penicillin 335 > 160 and 335 > 176 and, for novobiocin, 635 > 418 and 635 > 240. The method was validated according to the European Commission Decision 2002/657/EC (EC, 2002).

3.4 STATISTICAL ANALYSIS

Statistical analysis comprised descriptive analysis and significance testing ($p \leq 0.05$) of the difference between positive milk samples after the 7^{th} day and negative milk samples using logistic regression and Fisher's exact test (IBM SPSS Statistics, Version 22, 2013). The mastitis-causing pathogen, treatment interval, parity and cow status (milk production, fever and udder health) were evaluated. Nagelkerke R square and Hosmer & Lameshow Test were used to assess to what extent the data fits the model.

4. RESULTS

Table 12. Bacteria isolated from milk samples collected from mastitic quarters prior to initiation of treatment, in conjunction with information on the presence of antimicrobial residues in milk after completion of treatment and after the prescribed withhold period.
Preglednica 12. Izolirane bakterije iz vzorcev mleka, zbranih iz mastitičnih četrti pred začetkom zdravljenja, v povezavi z informacijami o prisotniosti protimikrobnih ostankov v mleku po končanem zdravljenju in po preteku predpisane karence.

Isolated bacteria	No (%) of cows	Number of positive cases					
		TTC	Neo	Amo	Cef	DHS	Gen
Streptococcus uberis	26 (39,4)	6	-	2	1	4	-
Escherichia coli	25 (37,9)	12	2	2	4	1	-
Klebsiella pneumoniae	4 (6,1)	4	1	-	-	-	-
Staphylococcus aureus	2 (3,0)	1	-	-	-	-	-
Enterococcus faecalis	2 (3,0)	1	-	-	-	-	-
Streptococcus dysgalactiae	1 (1,5)	-	-	1	-	-	-
Enterobacteriaceae	4 (6,0)	-	-	-	-	-	-
CNS	4 (6,0)	-	-	1	-	-	-
Candida spp.	2 (3,0)	-	-	-	-	2	-
No isolate	12 (18,1)	3	-	-	1	-	1

CNS - Coagulase Negative Staphylococci
TTC – tetracycline
Neo – neomycin
Amo – amoxicillin
Cef – cefquinome
Dhs – dihydrostreptomycin
Gen – gentamycin

We sampled milk from 66 cows before the beginning of the treatment for bacterial identification from mastitic quarters. In 12 (18.1%) samples no bacteria were isolated, the normal range of culture negative results (Guterbock et al. 1993; Sérieys et al., 2005). A total of 68 cultures were isolated and identified from 56 milk samples (Table 12). The gathered data from the questionnaire are shown in Table 13.

Table 13. Parity, number of affected quarters, treatment frequency per day and duration of treatment, WP in milkings, evaluation of milk production, fever, udder clinical signs (abnormal milk, milk clots, oedema), and isolated bacteria of cows treated for clinical mastitis.
Preglednica 13. Zaporedna laktacija, število prizadetih četrti, število zdravljenj na dan in trajanje zdravljenja, karenca izražena v molžah, ocena prireje mleka, zvišana telesna temperatura, klinične spremembe vimena (nenormalno mleko, mlečni strdki, vnetni edem vimena) in izolirane bakterije pri kravah, zdravljenih zaradi kliničnega mastitisa.

Cow No.	Parity	Affected quarters	Intramammary treatment			Decreased milk production	Fever	Abnormal milk/milk clots/oedema	Isolated bacteria
			Treatments per day	Duration (days)	WP in milking				
1	5	1	1	2	14	no	no	+ / - / +	*E. coli & K. pneumoniae*
2	3	1	1	6	12	++	+	+ / + / +	*E. coli*
3	6	2	1	5	10	no	no	- / + / -	*Str. uberis*
4	4	1	1	5	10	no	no	- / + / +	*Str. uberis*
5	1	1	1	5	10	no	no	- / + / +	*Str. uberis*
6	5	2	1	7	17	no	no	- / + /-	*Staph. aureus*
7	3	1	2	3	13	no	no	- / + / +	*E. coli*
8	1	1	2	3	13	no	+	+ / + / +	*E. coli; Str. uberis*
9	2	1	2	3	13	+	+	- / + / +	*E. coli*
10	5	1	1	5	14	+	no	+ / - / +	*E. coli*
11	3	1	1	5	12	++	+	+ / +/ +	*no isolate*
12	5	1	2	3	13	+	+	- / + / +	*no isolate*
13	6	1	2	3	13	no	no	- / + / -	*no isolate*
14	2	1	2	3	13	no	no	+ / + / +	*E. coli*
15	4	1	2	3	14	no	no	+ / - / +	*Str. uberis*
16	2	1	1	7	14	no	no	+ / + / -	*no isolate*
17	1	1	1	3	15	no	no	+ / - / +	*Str. uberis*
18	5	1	1	5	13	no	no	+ / - / +	*E. coli*
19	3	1	1	4	12	no	+	+ / - / +	*E. coli*
20	1	2	1	5	10	no	no	- / + / +	*Str. uberis*
21	5	1	1	5	12	no	no	+ / - / -	*no isolate*
22	1	2	1	5	10	no	no	+ / + / -	*Str. uberis*
23	4	2	1	5	10	no	no	- / + / -	*E. coli*
24	1	1	1	5	10	no	no	- / + / +	*Str. uberis*
25	2	1	1	5	12	+	+	+ / - / +	*no isolate*

Table 13 continues

Cow No.	Parity	Affected quarters	Intramammary treatment			Decreased milk production	Fever	Abnormal milk/milk clots/oedema	Isolated bacteria
			Treatments per day	Duration (days)	WP in milking				
26	1	1	2	3	14	+	+	- / + / +	*Str. uberis*
27	4	1	1	6	12	+	+	+ / - / +	*E. coli; Enterococcus faecalis*
28	2	1	2	3	13	no	+	- / + / +	*no isolate*
29	1	2	1	3	10	+	+	+ / + / +	*no isolate*
30	2	1	1	6	12	+	+	+ / - / +	*no isolate*
31	5	1	2	3	13	no	no	- / + / +	*Enterobacteriaceae*
32	3	1	2	3	14	+	no	- / + / +	*E. coli;* CNS*; Enterobacteriaceae*
33	1	1	2	4	13	no	no	- / + / +	*Str. uberis*
34	3	1	2	3	14	no	no	- / + / -	*no isolate*
35	5	2	1	5	12	no	no	- / + / -	CNS
36	1	1	1	5	8	+	no	+ / +/ +	*E. coli & K. pneumoniae*
37	3	3	2	2	8	no	no	+ / +/ +	*E. coli & K. pneumoniae*
37	3	3	2	2	8	no	no	+ / +/ +	not sampled
37	3	3	2	2	8	no	no	+ / +/ +	not sampled
38	2	1	1	5	8	no	no	- / +/ +	*E. coli & K. pneumoniae*
39	3	1	2	2.5	8	no	no	- / + / +	not sampled
40	2	1	1	5	8	+	no	+ / - / +	not sampled
41	5	2	1	4	8	no	no	- / + / -	not sampled
41	5	2	1	4	8	no	no	- / + / -	not sampled
42	2	1	2	2,5	8	no	no	- / + / +	not sampled
43	2	1	1	5	8	+	+	+ / +/ +	not sampled
44	2	1	1	5	8	+	+	+ / - / +	not sampled
45	3	1	2	2,5	8	no	no	+ / +/ +	not sampled
46	3	1	1	5	8	no	no	+ / +/ +	not sampled
47	2	1	1	5	8	no	no	- / + / +	not sampled
48	2	1	1	5	8	no	no	nr	not sampled
49	3	1	1	6	12	+	no	+ / +/ +	*Str. uberis*
50	1	1	2	3	13	no	no	- / + / +	*Str. uberis*
51	3	1	1	5	12	no	no	+ / - / +	*no isolate*
52	1	1	1	5	12	no	no	+ / +/ +	*Str. uberis & E. faecalis*

A. Siljanoski: The influence of treatment and health condition of the animal on drug excretion in bovine milk.
Ljubljana: UL, Veterinary faculty, 2020. Doctoral dissertation.

Table 13 continues

Cow No.	Parity	Affected quarters	Intramammary treatment			Decreased milk production	Fever	Abnormal milk/milk clots/oedema	Isolated bacteria
			Treatments per day	Duration (days)	WP in milking				
53	5	1	1	5	12	no	+	- / + / +	*Str. uberis*
54	1	1	1	/	14	no	no	- / - / +	*S. aureus*
55	4	2	1	nr	12	+	+	+ / +/ +	*Str. uberis*
56	2	1	1	3	13	no	nr	nr	*Str. uberis*
57	2	1	1	5	14	no	no	+ / - / +	*Str. uberis*
58	1	1	1	/	14	no	no	- / + / +	*Str. uberis*
59	3	1	1	4	14	+	no	+ / + / +	*Str. uberis*
60	6	1	2	3	14	no	no	+ / - / -	*E. coli*
61	4	1	1	5	6	+	no	+ / - / +	not sampled
62	3	1	1	5	6	+	no	+ / + / +	not sampled
63	2	2	1	5	6	no	no	- / + / -	not sampled
64	3	1	1	5	10	+	+	+ / + / +	not sampled
65	5	1	1	5	10	+	+	+ / + / +	not sampled
66	2	1	1	5	10	+	+	- / + / +	not sampled
67	1	1	1	5	6	+	no	+ / + / +	not sampled
68	2	1	1	5	10	+	+	+ / + / +	not sampled
69	1	1	1	5	14	no	+	nr	*E. coli & Str. uberis*
70	1	1	1	5	14	+	no	- / + / -	not sampled
71	3	1	1	/	14	+	+	+ / - / +	*E. coli*
72	4	1	2	/	14	+	nr	- / + / +	*E. coli*
73	6	1	1	5	14	no	+	+ / + / +	*E. coli*
74	1	1	1	/	14	+	no	+ / + / +	*Candida spp.*
75	6	1	1	/	14	+	no	+ / + / +	*Candida spp.*
76	2	1	1	5	14	+	no	- / - / +	*Str. uberis*
77	nr	1	2	2,5	14	+	+	+ / - / +	*Str. uberis*
78	2	1	2	2,5	14	no	no	+ / + / -	*Str. uberis*
79	1	1	2	2,5	14	no	no	- / + / +	*E. coli*
80	2	1	1	5	14	+	no	- / - / +	*Str. dysgalactiae*
81	2	1	1	5	14	no	no	+ / + / +	*E. coli &* CNS
82	2	1	2	2,5	14	no	no	- / + / -	not sampled
83	2	1	1	4	14	no	no	+ / + / -	not sampled

A. Siljanoski: The influence of treatment and health condition of the animal on drug excretion in bovine milk.
Ljubljana: UL, Veterinary faculty, 2020. Doctoral dissertation.

Table 13 continues

Cow No.	Parity	Affected quarters	Intramammary treatment			Decreased milk production	Fever	Abnormal milk/milk clots/oedema	Isolated bacteria
			Treatments per day	**Duration (days)**	**WP in milking**				
84	5	1	1		14	+	no	+ / + / +	*Str. uberis*
85		1	1	5	14	+	+	+ / + / +	no isolate
86	1	1	1	5	14	+	+	+ / + / +	*Str. uberis & Enterobacteriacea*
87	1	2	1	5	14	no	no	+ / + / +	not sampled
88	1	1	1	/	14	no	no	nr	not sampled
89	2	1	1	5	14	+	+	+ / + / +	not sampled
90		1	1	5	14	+	+	+ / + / +	not sampled
91	2	2	1	4	14	no	no	+ / + / -	not sampled
92	2	1	1	5	14	+	+	+ / + / +	not sampled
93	7	1	1	4	14	+	no	+ / + / +	not sampled
94	2	1	1	5	14	+	+	+ / + / +	*E. coli*
95	3	2	1	5	14	+	+	+ / + / +	*E. coli*
96	3	3	1	5	14	+	+	- / + / +	CNS
97	/	1	/	/	14	/	/	/	*E. coli*

CNS – Coagulase Negative Staphylococci
++ very decreased.
nr – not reported.

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Table 14. Intramammary and systemic products used for treatment of mastitis and prescribed WP in milkings in cows treated for clinical mastitis.
Preglednica 14. Uporabljeni intramamarni in sistemski pripravki pri posamezni kravi za zdravljenje kliničnega mastitisa in predpisana karenca izražena s številom molž.

Cow No.	Intramammary product						Systemic product				Imm product applied last day of treatment	Prescribed WP in milkings
1	Mastijet Fort						Hostamox	Baytril	Fynadine		Mastijet Fort	14*
2	Mastijet Fort	Cobactan					Cobactan	Enroxyl			Mastijet & Cobactan	12
3	Mastijet Fort		Klavuxil						Klavuxil		Mastijet & Klavuxil	10
4	Mastijet Fort		Klavuxil						Klavuxil		Mastijet & Klavuxil	10
5	Mastijet Fort		Klavuxil						Klavuxil		Mastijet & Klavuxil	10
6	Mastijet Fort		Klavuxil	Pb			Hostamox	Cobactan	Baytril	Dexa	All	17*
7	Mastijet Fort			Pb					Klavuxil		Mastijet & Pb	13
8	Mastijet Fort			Pb					Klavuxil		Mastijet & Pb	13
9	Mastijet Fort			Procapen				Enroxyl	Klavuxil		Mastijet & Procapen	13
10	Mastijet Fort	Cobactan	Klavuxil	Sustrepen	Gen	Dexa	Cobactan	Baytril	Fynadine		Mastijet Fort	14*
11	Mastijet Fort	Cobactan					Cobactan	Enroxyl			Mastijet & Cobactan	12
12	Mastijet Fort			Procapen				Enroxyl	Klavuxil		Mastijet & Procapen	13
13	Mastijet Fort			Procapen					Klavuxil		Mastijet & Procapen	13
14	Mastijet Fort			Procapen					Klavuxil		Mastijet & Procapen	13
15	Mastijet Fort			Procapen					Klavuxil		Mastijet & Procapen	14*
16	Mastijet Fort		Ubrolexin	Tetradelta	Gen			Baytril			All	14*
17	Mastijet Fort	Cobactan	Klavuxil	Sustrepen	Gen			Baytril			All	15*
18	Mastijet Fort	Cobactan					Cobactan	Enroxyl			Mastijet & Cobactan	13
19	Mastijet Fort	Cobactan					Cobactan	Enroxyl	Naklofen		Mastijet & Cobactan	12
20	Mastijet Fort		Klavuxil					Enroxyl			Mastijet & Klavuxil	10
21	Mastijet Fort		Klavuxil				Cobactan				Mastijet & Klavuxil	12
22	Mastijet Fort		Klavuxil						Klavuxil		Mastijet & Klavuxil	10
23	Mastijet Fort								Klavuxil		Mastijet Fort	10
24	Mastijet Fort		Klavuxil						Klavuxil		Mastijet & Klavuxil	10
25	Mastijet Fort	Cobactan					Cobactan	Enroxyl			Mastijet & Cobactan	12
26	Mastijet Fort			Procapen				Enroxyl	Klavuxil		Mastijet & Procapen	14*
27	Mastijet Fort	Cobactan					Cobactan	Enroxyl			Mastijet & Cobactan	12
28	Mastijet Fort			Procapen					Klavuxil		Mastijet & Procapen	13

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Table 14 continues

Cow No.	Intramammary product						Systemic product				Last imm product administered	Prescribed WP in milkings
29	Mastijet Fort	Cobactan					Cobactan	Enroxyl			Mastijet & Cobactan	10*
30	Mastijet Fort	Cobactan					Cobactan	Enroxyl			Mastijet & Cobactan	12
31	Mastijet Fort			Procapen					Klavuxil		Mastijet & Procapen	13
32	Mastijet Fort			Procapen				Enroxyl	Klavuxil		Mastijet & Procapen	14*
33	Mastijet Fort			Procapen				Enroxyl	Klavuxil		Mastijet & Procapen	13
34	Mastijet Fort			Procapen					Klavuxil		Mastijet & Procapen	14*
35	Mastijet Fort	Cobactan					Cobactan	Enroxyl			Mastijet & Cobactan	12
36	Mastijet Fort						Meloxidolor	Enroxyl	Klavuxil		Mastijet Fort	8
37	Mastijet Fort										Mastijet Fort	8
37	Mastijet Fort										Mastijet Fort	8
37	Mastijet Fort										Mastijet Fort	8
38	Mastijet Fort										Mastijet Fort	8
39	Mastijet Fort								Sustrepen		Mastijet Fort	8
40	Mastijet Fort						Engemicin				Mastijet Fort	8
41	Mastijet Fort										Mastijet Fort	8
41	Mastijet Fort										Mastijet Fort	8
42	Mastijet Fort						Engemicin			Mel	Mastijet Fort	8
43	Mastijet Fort							Enroxyl	Sustrepen		Mastijet Fort	8
44	Mastijet Fort							Enroxyl	Sustrepen		Mastijet Fort	8
45	Mastijet Fort								Sustrepen		Mastijet Fort	8
46	Mastijet Fort								Sustrepen		Mastijet Fort	8
47	Mastijet Fort						Engemicin				Mastijet Fort	8
48	Mastijet Fort								Sustrepen		Mastijet Fort	8
49	Cobactan						Cobactan				Cobactan	12
50			Pb				Klavuxil				Pb	13
51	Cobactan						Cobactan				Cobactan	12
52	Cobactan						Cobactan				Cobactan	12
53	Cobactan						Cobactan				Cobactan	12
54		Klavuxil		Tetradelta			Hostamox				All	14*
55	Cobactan						Cobactan	Enroxyl	Rapidexon		Cobactan	12
56				Procapen			Sustrepen				Procapen	13
57	Cobactan	Klavuxil	Paracef	Sustrepen			Cobactan	Enroxyl	Dinalgen	Trim	Paracef & Klavuxil	14*

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Table 14 continues

Cow No.	Intramammary product						Systemic product				Last imm product administered	Prescribed WP in milkings
58	Cobactan	Klavuxil	Pb	Sustrepen							All	14*
59	Ubrolexin	Klavuxil		Sustrepen				Enroxyl			All	14*
60				Procapen			Klavuxil				Procapen	14*
61				Tetradelta			Sustrepen			Mel	Tetradelta	6
62				Tetradelta			Sustrepen			Mel	Tetradelta	6
63		Klavuxil					Simivet R				Klavuxil	6
64	Ceffect						Klavuxil		Marbonor		Ceffect	10
65	Ceffect						Simivet R		Marbonor	Mel	Ceffect	10
66	Cefquinor						Sustrepen	Tolfine	Baytril		Cefquinor	10
67				Tetradelta			Sustrepen	Baytril		Mel	Tetradelta	6
68	Cefquinor						Klavuxil	Baytril		Mel	Cefquinor	10
69	Mastijet Fort						Sustrepen	Enroxyl		Mel	Mastijet Fort	14*
70	Mastijet Fort						Engemicyn			Mel	Mastijet Fort	14*
71	Mastijet Fort	Klavuxil	Ubrolexin	Sustrepen			Qivitan	Enroxyl	Baytril	Trim	Mastijet Fort	14*
72	Mastijet Fort										Mastijet Fort	14*
73	Mastijet Fort						Engemicyn				Mastijet Fort	14*
74	Mastijet Fort	Cobactan	Tetradelta	Sustrepen				Cobactan	Rapidexone		Mastijet Fort	14*
75	Mastijet Fort	Klavuxil	Ubrolexin	Sustrepen			Simivet R	Enroxyl	Rapodexone		Mastijet Fort	14*
76	Mastijet Fort						Simivet R				Mastijet Fort	14*
77		Klavuxil					Klavuxil	Baytril		Mel	Klavuxil	14*
78		Klavuxil					Klavuxil			Mel	Klavuxil	14*
79		Klavuxil					Klavuxil			Mel	Klavuxil	14*
80		Klavuxil					Klavuxil			Mel	Klavuxil	14*
81		Klavuxil					Klavuxil			Mel	Klavuxil	14*
82		Klavuxil					Simivet R			Mel	Klavuxil	14*
83	Ubrolexin	Klavuxil	Synulox	Sustrepen			Cobactan		Rapidexone		Klavuxil	14*
84	Ubrolexin		Synulox	Sustrepen				Baytril	Rapidexone		Synulox	14*
85				Tetradelta			Sustrepen			Mel	Tetradelta	14*
86				Tetradelta			Sustrepen	Baytril			Tetradelta	14*
87				Tetradelta			Klavuxil			Mel	Tetradelta	14*

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Table 14 continues

Cow No.	Intramammary product						Systemic product				Last imm product administered	Prescribed WP in milkings
88				Tetradelta			Sustrepen			Mel	Tetradelta	14*
89	Cefquinor						Cefquinor				Cefquinor	14*
90	Cefquinor						Cefquinor				Cefquinor	14*
91	Cobactan						Cobactan				Cobactan	14*
92	Cefquinor						Cobactan	Enroxyl		Mel	Cefquinor	14*
93	Cobactan	Synulox	Klavuxil	Sustrepen			Simivet R	Enroxyl	Baytril		Cobactan	14*
94	Cefquinor						Cobactan	Baytril		Mel	Cefquinor	14*
95	Cefquinor						Cobactan			Mel	Cefquinor	14*
96	Ceffect						Klavuxil	Enroxyl		Mel	Ceffect	14*
97	Cefquinor	Synulox	Klavuxil	Sustrepen			Cobactan	Baytril	Rapidexone	Fin	Cefquinor	14*

* - properly prescribed WP (44 out of 97 cows) by the veterinarians according to the Slovenian national legislation for off-label use of drugs in lactating cows.
Pb – Procaine benzylpenicillin
Dexa – Dexacortin
Gen – Gentamicin
Simivet R - Simivet Retard
Mel - Meloxidolor
Trim - Trimetogal
Fin - Finadine
WP – Withhold Period in milkings
Prescribed WP – Prescribed WP by the veterinarians

Table 15. List of applied intramammary products with its composition, WP in milkings, recommended treatment interval and duration according to Slovenian Summary Product Characteristics (SPC).
Preglednica 15. Seznam uporabljenih intramamarnih pripravkov z njihovo sestavo, karenco izraženo s številom molž, priporočeni intervali zdravljenja v dnevu in trajanje zdravljenja glede na slovenske povzetke glavnih značilnosti zdravila (SPC).

No	Intramammary product	Active ingredients	WP in milkings	Treatment interval	Treatment duration
1	Mastijet Fort	Tetracycline Neomycin Bacitracin Prednisolone	8	24 hour	Repeat if necessary
2	Cobactan	Cefquinome	10	12 hour	3 consecutive milkings
3	Ceffect	Cefquinome	10	12 hour	3 consecutive milkings
4	Cefquinor	Cefquinome	10	12 hour	3 consecutive milkings
5	Klavuxil	Amoxicillin Clavulanic acid	6	12 hour	3 consecutive milkings
6	Procapen	Procaine benzylpenicillin	12	no info	no info
7	Tetradelta	Pb Neomycin Novobiocin Dhs Prednisolone	6	24 or 48 hours	once
8	Ubrolexin	Cefalexin Kanamycin	10	24 hour	no info
9	Paracef	Cefoperazone	4	24 hour	no info
10	Pb	Procaine benzylpenicillin	12	24 hour	3 consecutive days
11	Gentamycin[1]	Gentamycin	14	no info	no info
12	Synulox*	Amoxicillin Clavulanic acid	7	12 hour	3 consecutive milkings

Pb – Procaine benzylpenicillin
Dhs – Dihydrostreptomycin
[1] – Gentamycin and Sustrepen (Table 16) were applied intramammarily despite they are intended for systemic application.
* - according to manufacturer's instructions

Table 16. List of used systemic products with their composition, WP in milkings, recommended treatment interval and duration according to Slovenian Summary Product Characteristics (SPC).
Preglednica 16. Seznam uporabljenih sistemskih pripravkov z njihovo sestavo, karenco izraženo s številom molž, priporočenimi intervali zdravljenja v dnevu in trajanje zdravljenja glede na slovenske povzetke glavnih značilnosti zdravila (SPC).

No	Systemic product	Active ingredients			WP in milkings	Treatment interval	Treatment duration
1	Cobactan 2.5%	Cefquinome			2	24 hour	3 – 5 days
2	Klavuxil	Amoxicillin		Clavulanic acid	2	24 hour	3 – 5 days
3	Hostamox	Amoxicillin trihydrate			6	48 hour	if necessary
4	Simivet Retard	Amoxicillin trihydrate			6	48 hour	until recuperation
5	Sustrepen	Procaine benzylpenicillin		Dihydrostreptomycin	8	24 hour	until recuperation
6	Enroxyl	Enrofloxacin			6	24 hour	3 – 5 days
7	Baytril	Enrofolxacin			10[1]	24 hour	3 days
8	Meloxidolor	Meloxicam (NSAID)			10	one application	/
9	Engemicin	Oxytetracycline			6	24 hour	3 – 5 days
10	Marbonor	Marbofloxacin			3	24 hour	3 – 5 days
11	Trimetogal	Sulfadimetoxine sodium	Trimethoprim 2.5%		no info	no info	no info
12	Dexacortin	Dexamethasone			6	one application	/
13	Rapidexon	Dexamethasone			6	one application	/
14	Dinalgen	Ketoprofen (NSAID)		Benzyl alcohol	0	24 hour	1 – 3 days
15	Fynadine	Flunixin (NSAID)		Phenol	2	24 hour	3 – 5 days
16	Naklofen	Diclofenac sodium (NSAID)			no info	no info	no info
17	Tolfine	Tolfenamic acid (NSAID)	Benzyl alcohol	Sodium formaldehyde sulphoxylate	2[2]	48 hour	if necessary

[1]After subcutaneous application.
[2]After intravenous application.

Twelve different antimicrobial drugs were used intramammarily and eleven for systemic application (Table 14, 15 and 16). Milk samples from 47 cows (Cow 1-35 and Cow 49-60) collected from untreated healthy quarters before and after the WP were free of antimicrobials.

We sampled milk from 100 milk quarters from 97 mastitis cows with mastitis (Table 13).

Milk sampled from 56 quarters from 53 cows (54.6%) had antimicrobial residues above the MRL after the prescribed WP by the veterinarians, 22 of them had residues after the 7th day – the WP required by the Slovenian National Legislation for off-lable use of drugs in lactating cows. In 53 off-label treated cows the veterinarians prescribed WP of less than 14 milkings. Thirtyone (58.5%) of them had residues above the MRL, whereas 5 of them (9.4%) after the 14th milking – a result acquired from the additional samples that we collected after the prescribed WP.

35 udder quarters from 59 cows treated with Mastijet Fort® contained tetracycline residues above MRL (100 µg/kg) after the prescribed WP by the veterinarians (Table 17), but in 13 of them (22%) was the MRL exceeded after the 7th day. The values of the positive samples before the prescribed WP of 14 milkings were extrapolated (Table 23) to 15th milking. Eleven (11.3%) milk samples from 9 cows contained tetracycline residues above the MRL in the 15th milking. All tetracycline positive samples contained residues of neomycin. Only in one out of 9 cases was the MRL for neomycin (1500 µg/kg) exceeded after the 7th day (Table 18). Neomycin was administered in all cows as a part of the tetracycline product (Matijet Fort, Intervet International, Boxmeer, the Netherlands).

Cefquinome was found above the MRL (20 µg/kg) in 4 (Table 19) out of 32 cows after the 7th day (21 cows treated with Cobactan, 8 with Cefquinor and 3 with Ceffect), amoxicillin was found above the MRL (4 µg/kg) in 6 out of 26 cows treated with Klavuxil (Table 20), gentamycin in 1 (Cow 16) out of 3 cases, and dihydrostreptomycin in 7 out of 12 cases treated with Sustrepen (Table 21).

One milk sample contained residues of tetracycline, neomycin and cefquinome above the MRL after the prescribed WP (Cow 10). Neomycin was present together with tetracycline, both above the MRL, in cow 10, 38, 40, 41, 43, 44, 45, 46, and 48. Cefquinome was present together with tetracycline in cow 2, 10, 49, 51, 64, and 66.

Table 17. Tetracyclin residues. Parity, number of affected quarters, treatment frequency per day and duration, prescribed WP in milkings, clinical signs (evaluation of milk production, fever, abnormal milk, milk clots, oedema), bacteria isolated and tetracycline concentration in milk of cows treated for clinical mastitis (MRL 100 µg/kg).

Preglednica 17. Ostanki tetraciklinov. Zaporedna laktacija, število prizadetih četrti, pogostost dajanja zdravila na dan in trajanje zdravljenja, predpisana karenca izražen s številom molž, klinični znaki (ocena prireje mleka, zvišana telesna temperatura, nenormalno mleko, mlečni strdki, vnetni edem vimena), izolirana bakterija povzročiteljica mastitisa in koncentracija tetraciklina v mleku krav po končanem zdravljenju in po predpisani karenci pri kravah zdravljenih za klinični mastitis (MRL 100 µg/kg).

Cow No.	Parity	Affected quarters	Intramammary treatment			Decreased milk production	Fever	Abnormal milk/milk clots/ oedema	Isolated bacteria	Tetracycline residues (µg/kg)					
										Milkings before and after prescribed WP					
			Treatments per day	Duration (days)	WP in milking					-2	-1	+1	+2	+3	+4
1	5	1	1	2	14	no	no	+ / - / +	*E. coli & K. pneumoniae*	343	42	17	**272**	n/s	n/s
2	3	1	1	6	12	++	+	+ / + / +	*E. coli*	759	531	**701**	**253**	n/s	n/s
3	6	2	1	5	10	no	no	- / + / -	*Str. uberis*	108	85	46	**143**	n/s	n/s
4	4	1	1	5	10	no	no	- / + / +	*Str. uberis*	668	670	**750**	**453**	n/s	n/s
5	1	1	1	5	10	no	no	- / + / +	*Str. uberis*	481	140	**297**	36	n/s	n/s
6	5	2	1	7	17	no	no	- / + /-	*Staph. aureus*	118	114	**111**	<5	n/s	n/s
7	3	1	2	3	13	no	no	- / + / +	*E. coli*	738	1037	**325**	**267**	n/s	n/s
8	1	1	2	3	13	no	+	+ / + / +	*E. coli & Str. uberis*	n/a	69	20	**115**	n/s	n/s
9	2	1	2	3	13	+	+	- / + / +	*E. coli*	2298	995	**718**	**593**	n/s	n/s
10	5	1	1	5	14	+	no	+ / - / +	*E. coli*	423	1032	**153**	83	n/s	n/s
11	3	1	1	5	12	++	+	+ / + / +	not isolated	1545	598	**749**	**352**	n/s	n/s
12	5	1	2	3	13	+	+	- / + / +	not isolated	621	263	99	**111**	n/s	n/s
13	6	1	2	3	13	no	no	- / + / -	not isolated	221	546	50	**267**	n/s	n/s
14	2	1	2	3	13	no	no	+ / + / +	*E. coli*	383	300	**244**	82	n/s	n/s
15	4	1	2	3	14	no	no	+ / - / +	*Str. uberis*	1598	1766	**462**	**125**	n/s	n/s
36	1	1	1	5	8	+	no	+ / + / +	*E. coli & K. pneumoniae*	n/s	4875	**344**	**519**	**2526**	**530**
37	3	3	2	2	8	no	no	+ / + / +	*E. coli & K. pneumoniae*	n/s	5415	**3104**	**669**	**735**	**256**

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Table 17 continues

Cow No.	Parity	Affected quarters	Intramammary treatment			Decreased milk production	Fever	Abnormal milk/milk clots/ oedema	Isolated bacteria	Tetracycline residues (µg/kg)					
			Treatments per day	Duration (days)	WP in milking					Milkings before and after prescribed WP					
										-2	-1	+1	+2	+3	+4
37	3	3	2	2	8	no	no	+ / + / +	not sampled	n/s	2344	**3181**	**1863**	**1184**	**298**
37	3	3	2	2	8	no	no	+ / + / +	not sampled	n/s	1418	**1245**	**1391**	**704**	**389**
38	2	1	1	5	8	no	no	- / + / +	*E. coli* & *K. pneumoniae*	n/s	1377	**2602**	**810**	**1123**	<5
39	3	1	2	2.5	8	no	no	- / + / +	not sampled	n/s	336	**1191**	**1007**	**436**	**135**
40	2	1	1	5	8	+	no	+ / - / +	not sampled	n/s	4761	**3713**	**2031**	**1643**	**627**
41	5	2	1	4	8	no	no	- / + / -	not sampled	n/s	5260	**3883**	**2680**	**3097**	**1997**
41	5	2	1	4	8	no	no	- / + / -	not sampled	n/s	4224	**2769**	**3546**	**2428**	**1961**
42	2	1	2	2,5	8	no	no	- / + / +	not sampled	n/s	851	**1116**	**367**	**687**	**126**
43	2	1	1	5	8	+	+	+ / + / +	not sampled	n/s	1745	**6057**	**1888**	**1452**	**1468**
44	2	1	1	5	8	+	+	+ / - / +	not sampled	n/s	574	**3058**	**1197**	**246**	**294**
45	3	1	2	2,5	8	no	no	+ / + / +	not sampled	n/s	14904	**6732**	**4986**	**2172**	**1543**
46	3	1	1	5	8	no	no	+ / + / +	not sampled	n/s	1997	**918**	**529**	**215**	<5
47	2	1	1	5	8	no	no	- / + / +	not sampled	n/s	82	56	**197**	<5	<5
48	nr	1	1	4	8	nr	nr	nr	not sampled	n/s	1617	**1540**	**732**	**691**	**164**
69	1	1	1	5	14	no	+	nr	*Str. uberis* & *E. coli*	1818	3330	**1147**	**992**	/	/
70	1	1	1	5	14	+	no	- / + / -	not sampled	227	155	**196**	**198**	/	/
71	3	1	1	5	14	+	+	+ / - / +	*E. coli*	668	554	**278**	**356**	/	/
72	4	1	2	/	14	+	no	+ / + / +	*E. coli*	1171	551	**439**	**n/s**	/	/

n/a – not analysed
nr – not reported
-2 – two milkings before WP.
-1 – one milking before WP.
+1 – one milking after WP.
+2 – two milkings after WP.
+3 – three milkings after WP.
+4 – four milkings after WP.
n/s – not sampled

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Table 18. Neomycin residues. Parity, number of affected quarters, treatment frequency per day and duration, WP in milkings, clinical signs (decreased milk production, fever, abnormal milk, milk clots, oedema,), isolated bacteria and neomycin concentration in milk of cows treated for clinical mastitis (MRL 1500 µg/kg).

Preglednica 18. Ostanki neomicina. Zaporedna laktacija, število prizadetih četrti, pogostost dajanja zdravila na dan in trajanje zdravljenja, predpisana karenca izražen s številom molž, klinični znaki (ocena prireje mleka, zvišana telesna temperatura, nenormalno mleko, mlečni strdki, vnetni edem vimena), izolirana bakteria povzročiteljica mastitisa in koncentracija neomicina v mleku krav po končanem zdravljenju in po predpisani karenci pri kravah zdravljenih za klinični mastitis (MRL 1500 µg/kg).

Cow No.	Parity	Affected quarters	Intramammary treatment			Decreased milk production	Fever	Abnormal milk/milk clots/ oedema	Isolated bacteria	Neomycin residues (µg/kg)					
			Treatments per day	Duration (days)	WP in milking					Milkings before and after prescribed WP					
										-2	-1	+1	+2	+3	+4
10	5	1	1	5	14	+	no	+ / - / +	*E. coli*	n/a	n/a	**2884**	**3730**	n/s	n/s
38	2	1	1	5	8	no	no	- / + / +	*E. coli* & *K. pneumoniae*	n/s	n/a	**1732**	278	511	<300
40	2	1	1	5	8	+	no	+ / - / +	not sampled	n/s	2264	**1652**	1366	**1654**	786
41	5	2	1	4	8	no	no	- / + / -	not sampled	n/s	3161	**2311**	**3061**	**3061**	**2816**
41	5	2	1	4	8	no	no	- / + / -	not sampled	n/s	2711	**2361**	**2536**	**2411**	**2361**
43	2	1	1	5	8	+	+	+ / +/ +	not sampled	n/s	n/a	**2366**	**1661**	1003	1069
44	2	1	1	5	8	+	+	+ / - / +	not sampled	n/s	n/a	**1827**	<300	<300	983
45	3	1	2	2,5	8	no	no	+ / +/ +	not sampled	n/s	n/a	1188	1055	1025	**1560**
46	3	1	1	5	8	no	no	+ / +/ +	not sampled	n/s	1758	**2138**	1461	<300	<300
48	nr	1	1	5	8	no	no	nr	not sampled	n/s	n/a	**1520**	832	757	<300

n/a – not analysed
nr – not reported
n/s – not sampled
-2 – two milkings before WP.
-1 – one milking before WP.
+1 – one milking after WP.
+2 – two milkings after WP.
+3 – three milkings after WP.
+4 – four milkings after WP.

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Table 19. Cefquinome residues. Parity, number of affected quarters, treatment frequency per day and duration, WP in milkings, clinical signs (decreased milk production, fever, abnormal milk, milk clots, oedema,), isolated bacteria and cefquinome concentration in milk of cows treated for clinical mastitis (MRL 20 µg/kg).

Preglednica 19. Ostanki cefquinoma. Zaporedna laktacija, število prizadetih četrti, pogostost dajanja zdravila na dan in trajanje zdravljenja, predpisana karenca izražen s številom molž, klinični znaki (ocena prireje mleka, zvišana telesna temperatura, nenormalno mleko, mlečni strdki, vnetni edem vimena), izolirana bakteria povzročiteljica mastitisa in koncentracija cefquinoma v mleku krav po končanem zdravljenju in po predpisani karenci pri kravah zdravljenih za klinični mastitis (MRL 20 µg/kg).

Cow No.	Parity	Affected quarters	Intramammary treatment			Decreased milk production	Fever	Abnormal milk/milk clots/oedema	Isolated bacteria	Cefquinome residues (µg/kg)					
			Treatments per day	Duration (days)	WP in milking					Milkings before and after prescribed WP					
										-2	-1	+1	+2	+3	+4
2	3	1	1	6	12	++	+	+ / + / +	*E. coli*	20-40	20-40	**20-40**	**20-40**	/	/
10	5	1	1	5	14	+	no	+ / - / +	*E. coli*	50	70	**55**	**60**	/	/
49	3	1	1	6	12	+	no	+ / +/ +	*Str. uberis*	/	/	<20	**20-30**	/	/
51	3	1	1	5	12	no	no	+ / - / +	*no isolate*	<30	<30	**20-30**	<20	/	/
64	3	1	1	5	10	+	+	+ / + / +	not sampled	/	/	**20-40**	**20-40**	**20-40**	**20-40**
66	2	1	1	5	10	+	+	- / + / +	not sampled	/	/	**<**20	**<**20	**20-40**	**<**20
90		1	1	5	14	+	+	+ / + / +	not sampled	40	20-30	**20-30**	<20	/	/
94	2	1	1	5	14	+	+	+ / + / +	*E. coli*	45	20-30	**20-30**	**20-30**	/	/
95	3	2	1	5	14	+	+	+ / + / +	*E. coli*	20-30	20-30	**20-30**	<20	/	/

Table 20. Amoxicillin residues. Parity, number of affected quarters, treatment frequency per day and duration, WP in milkings, clinical signs (decreased milk production, fever, abnormal milk, milk clots, oedema,), isolated bacteria and amoxicillin concentration in milk of cows treated for clinical mastitis (MRL 4 µg/kg).

Preglednica 20. Ostanki amoksicilina. Zaporedna laktacija, število prizadetih četrti, pogostost dajanja zdravila na dan in trajanje zdravljenja, predpisana karenca izražen s številom molž, klinični znaki (ocena prireje mleka, zvišana telesna temperatura, nenormalno mleko, mlečni strdki, vnetni edem vimena), izolirana bakteriia povzročiteljica mastitisa in koncentracija amoksicilina v mleku krav po končanem zdravljenju in po predpisani karenci pri kravah zdravljenih za klinični mastitis (MRL 4 µg/kg).

Cow No.	Parity	Affected quarters	Intramammary treatment			Decreased milk production	Fever	Abnormal milk/milk clots/oedema	Isolated bacteria	Amoxicillin residues (µg/kg)					
			Treatments per day	Duration (days)	WP in milking					Milkings before and after prescribed WP					
										-2	-1	+1	+2	+3	+4
77	/	1	2	2,5	14	+	+	+ / - / +	*Str. uberis*	22	29	**21**	**21**	ns	ns
78	2	1	2	2,5	14	no	no	+ / + / -	*Str. uberis*	22	19	**2**	**14**	ns	ns
79	1	1	2	2,5	14	no	no	- / + / +	*E. coli*	ns	2,5	**2**	**11**	ns	ns
80	2	1	1	5	14	+	no	- / - / +	*Str. dysgalactiae*	31	6	**20**	**6,1**	ns	ns
81	2	1	1	5	14	no	no	+ / + / +	*E. coli* & CNS	ns	28	**2**	**35**	ns	ns
82	2	1	2	2,5	14	no	no	- / + / -	not sampled	51	8,1	**7,9**	**145**	ns	ns
83	2	1	1	4	14	no	no	+ / + / -	not sampled	53	17	**<2**	3.1	ns	ns

Table 21. Dihydrostreptomycin residues. Parity, number of affected quarters, treatment frequency per day and duration, WP in milkings, clinical signs (decreased milk production, fever, abnormal milk, milk clots, oedema,), isolated bacteria and dihydrostreptomycin concentration in milk of cows treated for clinical mastitis (MRL 200 µg/kg).

Preglednica 21. Ostanki dihidrostreptomicina. Zaporedna laktacija, število prizadetih četrti, pogostost dajanja zdravila na dan in trajanje zdravljenja, predpisana karenca izražen s številom molž, klinični znaki (ocena prireje mleka, zvišana telesna temperatura, nenormalno mleko, mlečni strdki, vnetni edem vimena), izolirana bakteriia povzročiteljica mastitisa in koncentracija dihidrostreptomicina v mleku krav po končanem zdravljenju in po predpisani karenci pri kravah zdravljenih za klinični mastitis (MRL 200 µg/kg).

Cow No.	Parity	Affected quarters	Intramammary treatment			Decreased milk production	Fever	Abnormal milk/milk clots/oedema	Isolated bacteria	Dihydrostreptomycin residues (µg/kg)					
			Treatments per day	Duration (days)	WP in milking					Milkings before and after prescribed WP					
										-2	-1	+1	+2	+3	+4
57	2	1	1	5	14	no	no	+ / - / +	*Str. uberis*	2126	2120	**1538**	**361**	/	/
58	1	1	1	10	14	no	no	- / + / +	*Str. uberis*	1068	1068	**950**	**900**	/	/
59	3	1	1	4	14	+	no	+ / + / +	*Str. uberis*	2126	1302	**950**	**361**	/	/
74	1	1	1	/	14	+	no	+ / + / +	*no isolate*	2079	2008	**1726**	**1891**	/	/
75	6	4	1	/	14	+	no	+ / + / +	*no isolate*	1161	1741	**1361**	**997**	/	/
84	5	1	1	/	14	+	no	+ / + / +	*Str. uberis*	1538	1220	**1537**	**1102**	/	/
97	/	1	/	/	14	/	/	/	*E. coli*	1561	1350	**1526**	314	/	/

Logistic regression model (Table 22) was used to assess the impact of parity, treatments per day and decreased milk production on the likelihood that cows treated with tetracycline, cefquinome (including cow 64) and amoxicillin would have residues after the 7th day of the treatment (Table 14). In this analysis we included the extrapolated values (Table 23). The model explained 15.2% (Nagelkerke R square) of the variance in the cow status, and correctly classified 70.4% of the cases. The strongest predictor was treatmets per day, which was statistically significant ($p \leq 0.05$), followed by decreased milk production. Cows treated at 12 hour interval had higher chances for residues after the WP. Fever and udder's health (abnormal milk, milk clots and oedema) did not have significant effect on the excretion.

Table 22. Results from logistic regression model containing decreased milk production, treatments per day as categorical variables and parity as continuous variable.
Preglednica 22. Rezultati logističnega regresiskega modela, ki vsebuje zmanjšano proizvodnjo mleka, zdravljenja na dan kot kategorične spremenljivke in pariteto kot kontinuirano spremenljivko.

	B	S.E.	Wald	df	Sig.	Exp(B)	95% C.I.for EXP(B)	
							Lower	Upper
Hosmer and Lameshow test					.915			
Parity	.054	.161	.112	1	.738	1.055	.770	1.447
Treatments	-1.672	.570	8.612	1	**.003***	.188	.061	.574
DMP	-.579	.523	1.224	1	.269	.560	.201	1.563
Constant	.788	.750	1.102	1	.294	2.198		

*$p \leq 0.05$% statistically significant.
DMP – Decreased milk production.

The positive milk samples after the 7th day and the negative milk samples differed significantly ($p \leq 0.05$%; Fisher's exact test of independence) regarding *E.coli*. Cows infected with *E.coli* had higher risk of having residues after the 7th day.

In 12 samples we did not detect the cause of mastitis, 10 of them were free of antimicrobials. *Streptococcus uberis* was isolated from 26 cows, 17 of them did have residues after the 7th day, 3 had tetracycline, 2 amoxicillin (including 2 cows infected with *E. coli*) and 4 had dihydrostreptomycin.

5. DISCUSSION

In the present study, the farm veterinarians made the decisions for treatment and selection of the antimicrobial products. They used twelve different intramammary antimicrobial preparations (Table 15) and eleven systemic antimicrobial preparations (Table 16).

In all cows combination antimicrobial therapy was used. Off-lable was used in form of prolonged treatment instead the recommended, 24 hour interval treatment instead of 12 hour interval treatment and vice versa.

In 22 (22.7%) milk samples the concentration was above the MRL after the 7th day. In 13 milk samples tetracycline was detected above the MRL (100 μg/kg; Table 17), in 6 milk samples amoxicillin (4 μg/kg; Table 20), and in 4 cases cefquinome (20 μg/kg; Table 19). Dihydrostreptomycin was detected in 7 cases above the MRL (200 μg/kg; Table 21) and in one sample (Cow 16) gentamycin (MRL 100 μg/kg). Both dihydrostreptomycin (Sustrepen; GENERA d.d., Kalinovica, Croatia) and gentamicin were used intamammarily instead systemic as they are intended to be administered. Out of 12 cows treated intramammarily with Sustrepen, 7 (58.3%) milk samples had residues of dihydrostreptomycin above the MRL after the 14th milking (Table 21). These antimicrobials would have increased the percentage of positive milk samples after the 7th day by 7.2%. Because of their improper use, they were not included in the statistical analysis.

In 26 udder quarters from 23 (23.7%) off-label treated cows the MRL was exceeded before the 14th milking – the WP prescribed by the veterinarians. These include tetracycline (Table 17) and neomycin (Table 18) - both antimicrobials were administered by Mastijet Fort® (Mastijet Fort®, Intervet International, Boxmeer, the Netherlands), and Cefquinome (Table 19).

The MRL values of all positive samples that had prescribed WP by the veterinarians less than 14 milkings were extrapolated to 15th milking, the WP required by the Slovenian national legislation for drugs used off-label. Eleven milk samples from 9 cows had concentrations above the MRL in the 15th milking (Table 22). Taking into consideration these cows, the percentage of positive milk samples after the 7th day additionaly increased by 11%.

In some cases in our study bactericidal and bacteriostatic drugs were simultaneously administered intramammarily, for example in the case of tetracycline (bacteriostatic) and cefquinome (bactericidal) (Table 14). This combination is usually not practiced since its antagonistic effect.

All violations occurred as a result of intramammary treatment, none of the milk samples exceeded the MRL from systemic administration of the drugs. The MRL value was exceeded by six intramammary preparations after the 7th day. These comprised: Mastijet Fort® (Intervet International, Boxmeer, the Netherlands) in 13 out of 56 treated udder quarters, Klavuxil (GENERA d.d., Kalinovica, Croatia) in 6 out of 26 treated udder quarters, Cobactan LC (Intervet International, Boxmeer, the Netherlands) in 1 out of 21 treated udder quarters, Cefquinor LC (Norbrook Laboratories Limited, Newry, Northern Ireland) in 2 out of 8 treated udder quarters.

The treatment interval differed significantly ($p \leq 0.05$) between the positive milk samples after the 7th day and the negative milk samples treated intramammarily with tetracycline, cefquinome and amoxicillin (Klavuxil; GENERA d.d., Kalinovica, Croatia). The positive cows were also more likely to have decreased milk production and grater parity. Numerically, fever and udder changes (abnormal milk, clots in milk and oedema) were more frequent in the positive milk samples, but they were not strong predictors compared to the other sings. Combination antibiotic therapy, however, did not influence on the elimination kinetics of the antimicrobials. This result supports the passive diffusion concept of antimicrobial absorption by the udder (Gehring & Smith, 2006).

Escherichia coli and *Streptococcus uberis* were the most common microorganisms isolated from the infected quarters. Out of the total 24 cows infected with *E. coli* (without cow 97), 17 (68.0%) had residues after the prescribed WP. The infection with *E. coli* differed significantly ($p \leq 0.05$) between the positive and negative milk samples. *E. coli* is known to cause severe damage to the udder. It often results in death when the infection occurs in early lactation (FAO, 1989). Thirteen milk samples were positive on tetracycline (Table 17), two on amoxicillin (Cow 79 and 81) and one on cefquinome (Cow 95).

Streptococcus uberis was isolated from 24 quarters without *E. coli* and from 2 udder quarters with *E. coli*. Seven of the infected quarters (9 with *E. coli*) had antimicrobials. The average parity in the milk from cows negative to antimicrobials was 1.9, while in the positive 2.8.

In 12 cases no bacteria were found. Only two samples (cow 12 and 13) exceeded the MRL in the 15th milking. The average parity in the negative cows was 2.4, whereas in the postivie 4.3.

In seven samples the concentration of tetracycline was below the MRL in the first milking intended for consumption, while, at the next milking, the concentration of tetracycline was above the MRL. A similar finding has been reported by McEwen *et al.* (1992). When this occurs, a high value following a low value, for establishing the WP by the TTSC method the log-concentration values are substituted with their average (European Medicines Agency, 2000).

We are aware that in the present study the prescribed WP by the veterinarians in many off-label treated cows was shorter than that stated in the Slovenian National Legislation. However, our intention in this study was also to see all possible risks that could lead to antimicrobial residues in the bulk milk, without interfering with the decisions made by the veterinarians. In 53 off-label treated cows a WP of less than 7 days was prescribed. Twenty-eight milk samples were positive, whereas five of them still had residues above MRL after the 14thmilking. The values of the remaining 23 out of 45 positive milk samples were extrapolated to the 15th milking (Kissell et al., 2015). From the extrapolated values 11 milk samples from 9 cows still had residues above the MRL (Table 23) after the 14th milking.

The tetracycline residues had the highest concentrations in milk from a single udder quarter regarding the MRL - 67 and 60 times (Cow 45 and 43) above the MRL after the prescribed WP by the veterinarians. In both cases, five Mastijet Fort® preparations were administered, in one case at 12 hour interval (Cow 45) and in the other case at 24 (Cow 43) hour interval, instead of 3 times at 24 hour interval according to the specified product characteristics; and 4 times at 12 hour interval according to the manufacturer of the preparation. All cows treated with Mastijet Fort® with prescribed WP of 8 milkings had residues above MRL after the 8th milking, hence the high concentrations.

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Table 23. Extrapolation of antimicrobial residues concentration to 15 milking in cases when the WP was less than 14 milkings and the concentration of antimicrobial residues were exceeding the MRL value.
Preglednica 23. Preračunanje koncentracije protimikrobnih ostankov na 15 molž v primerih, ko je bila karenca manj kot 14 molž in so koncentracije ostankov presegale vrednost MRL.

Cow No.	Parity	Affected quarters	Intramammary treatment			Decreased milk production	Fever	Abnormal milk/milk clots/oedema	Extrapolated antimicrobial	Antimicrobial resides (µg/kg)				
			Treatments per day	Duration (days)	WP in milking					Milkings				
										11	12	13	14	15
2	3	1	1	6	12	++	+	+ / + / +	Tetracycline	759	531	701	253	**243**
36	1	1	1	5	8	+	no	+ / + / +	Tetracycline	2526	530	495	387	**303**
37	3	3	2	2	8	no	no	+ / + / +	Tetracycline	1184	298	296	178	**106**
37	3	3	2	2	8	no	no	+ / + / +	Tetracycline	704	389	358	261	**190**
38	2	1	1	5	8	no	no	- / +/ +	Tetracycline	1123	860	720	603	**504**
39	3	1	2	3	8	no	no	- / + / +	Tetracycline	436	135	201	152	**115**
40	2	1	1	5	8	+	no	+ / - / +	Tetracycline	1644	627	478	294	**181**
41	5	2	1	4	8	no	no	- / + / -	Tetracycline	3097	1997	1673	1348	**1085**
41	5	2	1	4	8	no	no	- / + / -	Tetracycline	2428	1961	1748	1480	**1253**
43	2	1	1	5	8	+	+	+ / +/ +	Tetracycline	1452	1468	1243	1041	**872**
45	3	1	2	2,5	8	no	no	+ / +/ +	Tetracycline	2172	1543	807	458	**260**

++ very decreased milk production

Enrofloxacin was analysed in samples before and after the WP by HPLC with a fluorescence detector and by STAR protocol. Enrofloxacin and its metabolite ciprofloxacin were detected in Cow 43, 44 and 64. The concentration of the sum of enrofloxacin and ciprofloxacin in all samples was less than 40 µg/kg, which was below the MRL (100 µg/kg). In our study enrofloxacin was always administered parenterally and its treatment ended earlier than intramammarily given preparations, hence its absence.

The presence of enrofloxacin in milk samples arising from the monitoring program in Croatia, was also reported by Bilandžić et al. (2011) with a maximum concentration of 24.3 µg/kg. Quinolones were detected in 20 samples taken from the Mexican market with concentrations ranging from 31.1 to 5047.3 µg/L (Ibarra et al., 2012). In pasteurized and UHT milk samples in China, 88.3% contained quinolones with a maximum concentration of 20.49 µg/kg (Zhang et al., 2014).

For the establishemt of WP for a particular product intended for intramammary treatment it is considered a worst case scenario as if all quarters are infected. In practice this situation is rare (EC, 2005). If we considered the concentration of residues in the total milk volume from one animal, it would decline due to the dilution with the milk from the other untreated quarters. Thus, the final concentration of the residues is expected to drop. Supposedly, if one quarter is treated, the final concentration of the drug in the total milk volume from one cow would be 25% of that of the milk from the treated quarter. Considering this, in 4 cases was the MRL for tetracycline exceeded (not including the extrapolated values from Table 23) after the 14th milking (Cow 9, 15, 69 and 72) in 4 cases was the MRL for amoxicillin exceeded (Cow 77, 80 81 and 82). Thus, almost one tenth of the treated animals had residues above the MRL after the 14th milking in the combined milk from all four quarters.

The concentrations of tetracycline from the treated quarters after the 14th milking were 593, 462, 1147 and 439 µg/kg, and the corresponding quantities in the milk from all four quarters exceeding the MRL value (100 µg/kg) were 148, 116, 287 and 110 µg/kg, respectively. In some cases (Cow 37 and 41) several quarters in the same cow were treated and in such cases we anticipate that the WP of 14 milking would not be enough. If we consider the extrapolated values from Table

23, cow 41 had the higest concentration of tetracycline after the 7th day. If mixed with the milk from other cows, it would be able to contaminate the milk from approximately 11 cows (Table 24), assuming that all cows produce the same amount of milk.

The presence of tetracyclines in bulk milk and in tank trailers was reported by Navratilova et al (2009). Tetracycline was identified in all analysed samples and, in 50.6% of samples oxytetracycline was also identified, although at levels far below the MRL. Tetracycline residues were detected at above the MRL in 0.34% of the samples in the Emilia-Romagna region, Italy (Serraino et al., 2013). In a study carried out in Croatia, 76% of milk samples were positive with respect to tetracycline (0 – 73.82 µg/kg); none exceeded the MRL (Vragović et al., 2011). In Brazil, on examination of pasteurized milk, 14% of the samples were found to contain oxytetracycline, one sample also contained tetracycline at a maximum content of 11.4 µg/kg (Spisso et al., 2010). In Turkey, 66.8% of analysed UHT milk samples contained tetracycline, at concentrations below the MRL (Unusan, 2009). In milk samples from China's market 1.7% of samples contained tetracycline at a maximum concentration of 47.7 µg/kg (Zhang et al., 2014).

The corresponding amoxicillin quantities in the milk from one cow after the 14th milking exceeding the MRL value (4 µg/kg) were 5, 5, 9 and 36 µg/kg in cows 77, 80, 81 and 82, respectively. All cows were treated 5 times instead the recommended 3 times at 12 hour interval. The intramammary preparation that contained amoxicillin (Klavuxil) has WP of 6 milkings in the label instructions, unlike the tetracycline preparation (Mastijet Fort®), which has longer WP of 8 milkings.

In Italy, Serraino et al. (2013) found, in 0.03% raw milk samples, β lactam antibiotics at concentrations exceeding the MRL values. In Croatia, during a three year study, two samples of 1259 were positive on penicillins (Bilandžić et al., 2011).

Neomycin was detected in all tetracycline positive samples. Neomycin was administered as part of the Mastijet Fort® (Intervet International, Boxmeer, the Netherlands). In ten milk samples (Table 18) was the concentration of neomycin above the MRL after the prescribed WP by the veterinarians. Taking into consideration the whole milk from a cow, the concentration of neomycin will decline below the MRL in all cases.

Neomycin was detected in 41 out of 1259 raw milk samples during national monitoring in Croatia (Bilandžić et al., 2011). The highest concentration detected was 1453.3 µg/kg.

Table 24. Bulk milk tank volume contaminated with tetracycline (MRL 100 µg/kg) supposing that milk from one treated cow enters the bulk milk tank. In calculation we assumed that one quarter was treated and that each quarter produces equal amount of milk - ca. 20 litres per cow.
Preglednica 24. Prikaz količine kontaminiranega mleka s tetraciklinom, ki bi preseglo vrednost MRL (100 µg/kg) v primeru, da bi zašlo kontaminirani mleko ene krave v cisterno; pri izračunu smo predpostavili, da je bila zdravljena ena četrt in da je bila proizvodnja mleka enaka iz vsake četrti, skupaj 20 litrov na kravo

TC in milk from 1 quarter (µg/kg)	TC in milk from a single cow (µg/kg)	Liters of contaminated milk
6000	1500	300
5750	1438	280
5500	1375	260
5250	1313	260
5000	1250	240
4750	1188	220
4500	1125	220
4250	1063	200
4000	1000	200
3750	938	180
3500	875	160
3250	813	160
3000	750	140
2750	688	120
2500	625	120
2250	563	100
2000	500	100
1750	438	80
1500	375	60
1250	313	60
1000	250	40
750	188	20
500	125	20
400	100	20

The WP is evaluated by the manufacturer of the drug preparation, nevertheless it can differ between countries. According to the European Medicinal Agency the harmonised method in the EU to determine a WP is the 'Time to Safe Concentration' (TTSC). Using healthy dairy cows, it determines the time necessary the concentration to drop below, and remain below the MRL (European Medicines Agency, 2000). However, the pharmacokinetics of antimicrobials administered to cows with mastitis differ from those of healthy animals (Cagnardi et al., 2010). During mastitis the pH of milk often increases. An antimicrobial may pass through the epithelial barrier by passive diffusion. Only unbound, nonionized and lipid soluble drugs pass through the udder's epithelium into the plasma (Ziv & Sulman, 1975; Ghering & Smith, 2006). The prolonged excretion of residues in milk, as shown in our results, can be partly attributed to the physical and chemical changes in the mastitic mammary gland. In clinical experiment, which is necessary for determination of WP, the prescribed preparation is applied intramammarily to all four quarters. From our results we can notice that, the probability of having antmicriobial residues in cows is greater when we treat more than one quarter in a single cow.

For monitoring of antimicrobial residues in milk two types of screening tests are mainly used: inhibition of microbial growth and rapid tests (immunoassays). There is no screening test that will satisfy all requirements and detect all antimicrobials used in dairy cattle. The screening tests used by farmers, mostly, are aimed at penicillins (Mitchell et al., 1998). Many screening tests are capable of detecting only single antimicrobial family. Twinsensor (Unisensor S.A., Belgium), beside penicillins and cephalosporins, can detect tetracyclines, but not other class of antimicrobials used in dairy cows. Penicillins are still a major class of antibiotics used in the mastitis treatment. In our study we detected 6 antimicrobials (tetracycline, neomycin, cefquinome, amoxicillin, dihydrostreptomycin and gentamicin) with concentration above the MRL. The 5 plate method is able to detect all of them within the limit, except for cefquinome (MRL 20), which can be detected slightly above the MRL at 20-30 µg/kg. However, the 5 plate method is not a preferable method on a farm, given the long procedure and the need of laboratory equipment and a trained analyst. Delvotest® SP-NT is preferable to use on a farm but it is not sensitive to tetracycline (270 - 320

µg/kg; Technical Bulletin, DSM Food Specialties, The Netherlands) and cefquinome (65-75 µg/kg).

Treatment in dairy cows often includes other classes of antimicrobials such as quinolones, aminoglycosides or sulphonamides. Most importantly, farmers must withhold the milk during the WP to ensure antimicrobial free milk. When larger number of cows are treated in the herd, we recommend antimicrobial analysis of the milk, before it goes to the bulk milk tank. The screening tests used should be able to detect all antimicrobials applied in the treatment.

6. CONCLUSIONS

Hypothesis 1. The excretion of drugs in milk from cows with mastitis and cows with repeated mastitis is different compared to healthy cows.

From the acquired milk samples 22 out of 97 cows (22.7%) had antimicrobial residues above MRL after the 7th day of the last treatment. The positive cows had greater probability to have decreased milk production and grater parity. Numerically, fever and udder changes (abnormal milk, clots in milk and oedema) were more often present in the positive milk samples, but they were not strong predictors as the other sings.

The infection with *E. coli* differed significantly ($p \leq 0.05$) between the positive and negative milk samples. *E. coli* was isolated from 24 cows (cow 97 was excluded from the analysis), in which 17 (70.8 %) of them had residues above MRL. Decresed milk production was twice more frequent in the positive cows.

Additionally, intramammary treatment with preparations not intended for intramammary application, like in the case with Sustrepen and gentamicin, represents a safety hazard because of the high percentage of positive milk samples after the 7th day of last treatment.

If milk from several post treatment cows is consigned for sale along with milk from untreated cows then milk quality and safety may be at risk. Withhold periods only apply to individual cows. When larger number of dairy cows in the herd are treated, taking into consideration the occurrence of prolonged excretion of antimicrobials, it would be prudent to test the milk before it goes to the bulk milk tank.

Hypothesis 2. Off-label use of antimicrobial substances, as well as the use of different preparations simultaneously, may result in prolonged excretion of antimicrobials in milk.

The treatment interval differed significantly ($p \leq 0.05$) in the positive milk samples and the negative milk samples treated with tetracycline, cefquinome and amoxicillin. Cows treated at 12 hour

interval had greater chances of having residues after the 7th day of the treatment. There was no crucial difference between the positive and the negative cows regarding the combination antibiotic therapy.

Hypothesis 3. Antimicrobial substances used for treatment of cattle cannot be detected by one screening test only.

One of the main goals of each screening test is to give accurate results within a short period of time. There is no suitable screening test to cover all the antimicrobials used on a farm. Delvotest SP-NT test is widely used since the large range of antimicrobial detection in milk. However, it is not able to detect tetracycline, cefquinome and dihydrostreptomycin within the safety limit. Other classes of antimicrobials such as quinolones and sulphonamides must not be neglected, despite their systemic use. In order to cover wider spectrum of antimicrobials used in dairy cattle, using more than one screening tests simultaneously should be considered.

7. SUMMARY

Causes of extended excretion of antimicrobials in milk after intramammary treatment can be a result of prolonged or increased dosage, decreased milk production and physiological changes in the udder caused by the infectious agent. Low-producing cows have a high tendency to exceed MRL in milk than high-producing cows. High-producing cows dilute and eliminate the drug fast enough leaving shorter time for the drug to interact with the microorganism, thus lowering the clinical efficacy. The concentration of drugs in milk after intramammary treatment is also affected by the absorption rate of the drug from the udder epithelia, which depends on the lipid solubility of the undissociated molecule. The purpose of this study was to determine, on commercial farms, if treated clinical mastitis requires longer WP, while the treatment decisions were made by the veterinarians only. Additionally, we scrutinized which screening test is suitable for detection of all antimicrobials used in the treatment.

In the present study 97 cows with clinical mastitis were included. Milk samples were collected from 100 inflamed udder quarters. Additionally, 48 milk samples were taken from untreated healthy udder quarters to check if any crossover of drugs had occurred. Milk samples from 66 affected udder quarters were taken for bacterial identification before treatment. All lactating cows were treated by a combination of drugs and/or off-label. Combination antimicrobial therapy was used in all cases, prolonged treatment instead the recommended, different route of administration not suggested in the label instructions as well as 12 hour interval treatment instead of 24 hour interval treatment and vice versa. The attending veterinarians completed a questionnaire, providing data about the cow; prescribed WP, lactation number, number of infected udder quarters, clinical signs (fever, quarter oedema, decreased milk production, abnormal milk); and applied treatments.

For antimicrobial detection three screening tests were used. The positive results were analysed with the appropriate confirmatory method. Tetracyclines were analysed with LC-MS/MS, penicillins with UPLC-MS/MS and enrofloxacin together with ciprofloxacin with HPLC-

fluorescence detector. Neomycin, cefquinome, dihydrostreptomycin and gentamycin were quantified with the 5-Plate method, so called the STAR protocol.

Twelve different intramammary preparations and eleven preparations for systemic application were used. All samples exceeding the MRL value were a result of intramammary applications, none of the milk samples exceeded the MRL from systemic administration of antimicrobial drugs. The treatment with systemic preparations was always shorter, hence the negative result. All samples taken from the untreated healthy quarters were free of antimicrobial residues before and after the prescribed WP. The intramammary preparations that had prolonged excretion were: Masitjet Fort® in 13 cows, Cobactan in 1, Cefquinor in 2 and Klavuxil in 6 cows. Violations were noted in 22 (22.7%) cows after the 14^{th} milking. Dihidrostreptomycin and gentamicin were used intramammarily instead of parenteraly as they are intended. In 7 cases the values of dihydrostreptomycin and in 1 case of gentamicin were above the MRL after the 7^{th} day of the last treatment. If we considered the concentration of residues in the total milk volume from one cow, in 4 cases was the MRL for tetracycline and amoxicillin exceeded.

Escherichia coli and *Streptococcus uberis* were the most common microorganisms isolated from the infected quarters. From 24 cows infected with *E. coli*, 17 (68.0%) had residues after the prescribed WP. The infection with *E. coli* was significant ($p \leq 0.05$) in positive compared to negative milk samples. Among the cows infected with *Streptococcus uberis* larger difference in parity ($p \leq 0.10$) was noted between the positive and the negative cows.

The treatment interval differed significantly ($p \leq 0.05$) between the positive and the negative milk samples treated with tetracycline, cefquinome and amoxicillin. Cows treated at 12 hour interval have greater risk of having residues after the 7^{th} day of the last treatment. Combination antibiotic therapy, however, did not influence on the elimination kinetics of the antimicrobials.

The WP is evaluated by the manufacturer of the drug preparation, nevertheless it can differ between countries. According to the European Medicinal Agency the harmonised method in the EU to determine a WP is the 'Time to Safe Concentration' (TTSC). Using healthy dairy cows, it determines the time necessary the concentration to drop below, and remain below the MRL. During mastitis the pH of milk often increases. An antimicrobial may pass through the epithelial barrier

by passive diffusion. Only unbound, nonionized and lipid soluble drugs pass through the udder's epithelium into the plasma. The prolonged excretion of residues in milk, as shown in our results, can be partly attributed to the physical and chemical changes in the inflamed mammary gland.

Sale of milk that contains antimicrobial drug residues is illegal, however no specific official screening tests have been specified. One of the main goals of each screening test is to give accurate results within a short period of time. On-site screening for antimicrobials is recommended but not obligatory. There are many commercially available screening tests, but no one is capable to cover all the antimicrobials used on a farm. Delvotest SP-NT test is widely used because of the high sensitivity to numerous antimicrobials, however his downside is the very low specificity. In our study it was not able to detect tetracycline, cefquinome and dihydrostreptomycin within the safety limit. Other classes of antimicrobials such as quinolones and sulphonamides must not be neglected, despite their systemic use. At choosing a screening test on a farm it is reasonable to take into consideration the antimicrobials used or it should be based on the predictive values obtained from preliminary studies on the type of antimicrobials and prevalence of residues in the area.

8. POVZETEK

Pri zdravljenju vnetja vimena, mastitisu, se soočamo s podaljšanim izločanjem antimikrobov v mleku, predvsem pri intamamarnem dajanju zdravil. Vzroki podaljšanega izločanja so različni, na primer podaljšan čas dajanja zdravila, kar ima za posledico povečan odmerek, zmanjšana količina mleka in fiziološke spremembe v vimenu, ki nastanejo zaradi okužbe z bakterijami. Pri kravah, ki proizvajajo dnevno manj mleka, je večja verjetnost, da bo vrednost MRL uporabljene učinkovine presežena, kot pri kravah, ki proizvajajo velike količine mleka. Pri takih kravah se namreč zdravilo bolj razredči in tudi hitreje izloči, zaradi tega pa deluje krajši čas in je posledično manj učinkovito. Na koncentracijo zdravil v mleku po intramemarnem zdravljenju vpliva tudi absorpcija zdravil skozi epitelij vimenskih četrti, ki je odvisna od topnosti nedisocirane molekule v lipidih. Namen raziskave je bil določiti ostanke antimikrobnih zdravil v mleku krav molznic, na komercijalnih farmah, če zdravljenih kravah s mastitom zahtevajo daljšo karenco, medtem so odločitve o zdravljenju sprejemali samo veterinarji. Poleg tegam smo pregledali, kateri presejalni test je primeren za detekcijo vseh protimikrobni zdravil, ki se uporabljaju pri zdravljenju.

V raziskavo je bilo vključenih 97 krav molznic s kliničnim mastitisom. Vzorci mleka so bili zbrani iz stotih okuženih vimenskih četrti. Večina krav je imela okuženo po eno četrt, nekaj krav pa po dve ali tri četrti. Vsi vzorci mleka so bili pregledani na morebitno vsebnost zdravil. Poleg tega je bilo zbranih tudi 48 vzorcev mleka iz zdravih, nezdravljenih vimenskih četrti, ki so bili pregledani na morebitno vsebnost zdravil. Za identifikacijo bakterij so bili odvzeti vzorci mleka pred zdravljenjem iz 66 mastitičnih vimenskih četrti. Vse molznice so bile zdravljene s kombinirano antimikrobno terapijo, v nekaterih primerih so se posluževali izjemne uporabe zdravil in sicer podaljšanega dajanja namesto predpisanega, drugačnega vnosa od predpisanega v navodilih za uporabo, neupoštevan priporočen časovni interval dajanja zdravil. Lečeči veterinarji so izpolnili vprašalnik in v njem podali informacijo o karenci, ki so jo predpisali, o zaporedni laktaciji krave, o številu okuženih vimenskih četrti, o osnovni klinični sliki (vročina, edem v vimenskih četrtih, količina mleka, izgled mleka) in trajanju zdravljenja. Kljub slovenski zakonodaji za izjemno uporabo zdravil, ki opredeljuje karenco najmanj 14. molž, je bila predpisana karenca s strani

lečečih veterinarjev pri 54. kravah (od 97) krajša.

Za detekcijo antimikrobov so bili uporabljeni trije presejalni testi in sicer DELVO test, Tween sensor in metoda petih plošč imenovana tudi STAR protokol. Le z uporabo vseh omenjenih presejalnih testov so bili zajeti vsi antimikrobi, ki so jih uporabljali lečeči veterinarji. Pozitivni vzorci so bili analizirani še z ustrezno potrditveno metodo, s katero smo natančneje identificirali antibiotik in ovrednotili njegovo vsebnost v mleku. Tetraciklini so bili analizirani z LC-MS/MS, penicilini z UPLC-MS/MS in enrofloksacin skupaj s ciprofloksacinom s HPLC-fluorescenčnim detektorjem. Neomicin, cefkvinom, dihidrostreptomicin in gentamicin so bili kvantificirani z uporabo selektivnih plošč za posamezni antibiotik, ki jih uporabljamo pri STAR protokolu.

Za zdravljenje mastitisa je bilo uporabljenih dvanajst intramamarnih pripravkov in enajst pripravkov za parenteralno dajanje. Vsi vzorci mleka, v katerih so bile najdene vsebnosti antimikrobov nad MRL-vrednostmi, so bili posledica intramamarnega dajanja zdravil. Vzorci mleka, zbrani iz zdravih vimenskih četrti pred in po predpisani karenci niso vsebovali antimikrobnih ostankov zdravil. V mleku iz zdravljenih četrti pa smo po sedmih dnevih karence ugotovili ostanke antimikrobov pri uporabi sledečih intramamarnih pripravkov: Masitjet Fort® (tetraciklin) v 13 primerih, Cobactan (cefkvinom) v enem, Cefquinor (cefkvinom) v 2 primerih, in Klavuxil (amoksicilin) v 6 primerih. Preseženo MRL-vrednost smo opazili pri 22 kravah (22.7%) s predpisano karenco 14. molž. Dihidrostreptomicin in gentamicin sta bila uporabljena intramamarno, čeprav je bilo v navodilu predvideno parenteralno dajanje. Ostanke dihidrostreptomicina smo ugotvili v 7 primerih od 12, gentamicina pa v enem od 3.

Če pa upoštevamo koncentracijo ostankov v skupni količini mleka ene krave, potem je bila presežena mejna vrednost ostankov za tetraciklin in amoksicilin v 4 primerih.

Najpogostejša mikroorganizma, izolirana iz okuženih vimenskih četrti, sta bila *Escherichia coli* in *Streptococcus uberis*. Pri 24. kravah, okuženih z *E. Coli,* smo kar v 17. (68%) primerih ugotovili ostanke zdravil v mleku po preteku karence. Pokazalo se je, da je okužba vimena z *E. coli* signifikantno vplivala ($p \leq 0.05$) na prisotnost ostankov zdravil v mleku. Pri kravah okuženih s *Streptococcus uberis* je število telitev vplivalo ($p \leq 0.10$) na prisotnost zaostankov v mleku, pri kravah z več telitvami je bila večja verjetnost da so bili antimikrobni zaostanki prisotni v mleku.

Časovni interval dajanja zdravil se je signifikantno ($p \leq 0,05$) razlikoval med pozitivnimi in negativnimi kravami po karenci sedmih dni pri zdravljenju s tetraciklinom, cefkininom in amoksicilinom. Pri kravah, ki so dobivale odmerke v 12-urnem intervalu, je bilo večje tveganje, da bodo po 7. dnevu karence ostanki zdravil še prisotni v mleku. Kombinirana terapija, intramamarno in parenteralno dajanje istočasno, pa ni vplivala na izločanje antibiotikov z mlekom.

Karenco določa proizvajalec pripravka in je lahko različna glede na državo. Evropska agencija za zdravila je za Evropsko Unijo predpisala standardizirano metodo za določanje karence - Time to Safe Concentration (TTSC). Zdravilo se aplicira zdravim kravam molznicam in se določa potreben čas, da se koncentracija ostankov zdravil zniža pod dovoljeno MRL-vrednost ter se jo vzdržuje pod MRL. Pri mastitisu se vrednost pH mleka zviša. Antimikrobna zdravila lahko preidejo skozi epitelno bariero s pasivno difuzijo. Le nevezana, neionizirana in v lipidih topna zdravila preidejo skozi epitelij vimenske četrti v plazmo. Rezultati naše raziskave so pokazali, da na podaljšano izločanje antimikrobnih ostankov v mleko delno vplivajo fiziološke in kemijske spremembe v vnetih mlečnih žlezah.

Prodaja mleka, ki vsebuje antimikrobne zaostanke je nelegalna. V mlečno predelovalnih obratih izvajajo nadzor nad zaostanki zdravil v mleku, V ta namen uporabljajo hitre presejalne teste, ki pa niso predpisani. Eden od glavnih namenov presejalnega testa je dobiti v kratkem času točne rezultate. Kontrola za prisotnost antimikrobnih zaostankov na farmi je priporočljiva, ni pa obvezna. Veliko presejalnih testov je dostopnih v prosti prodaji, vendar noben nima zmožnosti, da bi določil vse antimikrobne snovi, ki se uporabljajo pri zdravljenju. Delvotest SP-NT se veliko uporablja, predvsem zaradi velike občutljivosti na številne antimikrobne snovi, vendar pa je njegova slabost nizka specifičnost. V naši raziskavi z Delvotestom SP- NT nismo določili tetraciklin, cefkvinom in dihidrostreptomicin v koncentracijah blizu MRL-vrednosti. Nikakor se ne sme zanemariti tudi druge skupine antimikrobov, kot so kinoloni in sulfonamidi, čeprav jih uporabljamo le parenteralno. Pri izbiri presejalnih testov na farmi bi bilo zaželjeno, da se uporabi test, ki je občutljiv na uporabljeni antimikrob pri zdravljenju mastitisa. Sicer naj bi izbira presejalnega testa temeljila na podatkih dobljenih od predhodne študije za uporabo antimikrobov in prevalenco ostankov na tem območju.

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11. APPENDIX

APENDIX A: Questionnaire for farm veterinarians

<table>
<tr><td colspan="6">Ime farme</td></tr>
<tr><td colspan="3">Podatki o vzorcu, številka primera:</td><td colspan="3"></td></tr>
<tr><td>Starost živali</td><td></td><td rowspan="5">Datum (jutranja/ večerna) odvzema vzorca</td><td rowspan="2">Molža</td><td colspan="2" rowspan="2">Zdravljenih krav v laktaciji
1x vzorec iz Bolna</td></tr>
<tr><td>Zaporedna laktacija</td><td></td></tr>
<tr><td>Dnevna proizvodnja mleka</td><td rowspan="2"></td><td></td><td colspan="2"></td></tr>
<tr><td rowspan="2">Dan laktacije</td><td></td><td colspan="2"></td></tr>
<tr><td></td><td colspan="2"></td></tr>
<tr><td colspan="6">Klinični znaki:</td></tr>
<tr><td colspan="2">Padec mleka (+/-)</td><td></td><td colspan="3">Abnormalno mleko (obkroži spodaj)</td></tr>
<tr><td colspan="2">Hipertermija (+/-)</td><td></td><td colspan="3">Rumeno – Rdeče – Vodeno</td></tr>
<tr><td colspan="2">Povečana četrt (+/-)</td><td></td><td colspan="3" rowspan="2">Druge spremembe (dopiši)</td></tr>
<tr><td colspan="2">Kosmiči v mleku (+/-)</td><td></td></tr>
<tr><td colspan="2">Vrsta okužbe (povzročitelj)</td><td colspan="4"></td></tr>
<tr><td colspan="6">Terapija</td></tr>
<tr><td colspan="2">Način vnosa</td><td colspan="2">INTRAMAMARNO</td><td colspan="2">PARENTERALNO</td></tr>
<tr><td colspan="2">Zdravilo (komercialno ime)</td><td colspan="2"></td><td colspan="2"></td></tr>
<tr><td colspan="2">Odmerek (doza)</td><td colspan="2"></td><td colspan="2"></td></tr>
<tr><td colspan="2">Trajanje in čas zdravljenja</td><td colspan="2"></td><td colspan="2"></td></tr>
<tr><td colspan="2" rowspan="2">Zdravljena četrt (B – bolna ali Z - zdrava)</td><td>pL</td><td>pD</td><td colspan="2" rowspan="2">i/m; s/c; i/v</td></tr>
<tr><td>zL</td><td>zD</td></tr>
<tr><td colspan="2">Karenca (v molži)</td><td colspan="4"></td></tr>
</table>

Odvzem vzorca mleka:
0) En vzorec pred zdravljenje za detekcijo bakterije.
Oznaka vzorcev:
0 (za detekcijo povzročitelja) – Ime farme – Ušesna številka živali - Četrt.
B (bolna četrta) število molže po zdravljenja – Ime Farme – Ušesna št. živali.
*Sterilne epruvete za bakteriološko preiskavo se dajejo v hladilnik (1-4°C).
*Epruvete za določanje antibiotikov se dajejo v zamrzovalnik (-20°C).