SELECTION OF ANTIMICROBIAL AGENTS

The number of antimicrobial agents that can be applied in a routine test is limited. To make testing relevant and practical, it makes sense to include those antibiotics for which commercial preparations are available on the market. In most antibiotic groups, representative molecules can be selected with the same spectrum of activity and full cross-resistance. This approach can deliver reliable and consistent results. In some antibiotic classes, such as the aminoglycosides, this is not possible. All molecules of this group show incomplete cross-resistance. However, in general, testing each antimicrobial available on the market is not necessary.

In addition to choosing representatives of antibiotic classes, discs that test for specific types of antimicrobial resistance should also be added. An example of this is cefoxitin for MRSA detection in Staphylococcus spp. and cefotaxime for ESBL detection in Enterobacteriaceae.

Antimicrobial classes

Only those antimicrobial classes that are relevant in the treatment of mastitis are mentioned in this manual.

Penicillins

- **Narrow-spectrum, β-lactamase-susceptible penicillins** are primarily active against non-β-lactamase-producing, Gram-positive bacteria such as streptococci and staphylococci. They also act against some fastidious bacteria such as Pasteurella. This group consists of: benzathine penicillin, benzyl penicillin (pen G), procaine penicillin and penethamate. Susceptibility is tested with a penicillin (1 unit) disc.

- **Narrow-spectrum, β-lactamase-resistant penicillins** are also active against Gram-positive bacteria such as streptococci and staphylococci, including the β-lactamase-producing Staphylococcus spp. This group consists of cloxacillin, dicloxacillin, flucloxacillin, methicillin, nafcillin and oxacillin. Susceptibility of staphylococci is tested with a cefoxitin disc (30 µg), that of streptococci with an oxacillin disc (1 µg).

- **Moderate-spectrum penicillins** (ampicillin and amoxicillin) are active against streptococci, non-β-lactamase-producing staphylococci as well as against Gram-negative species, including some members of Enterobacteriaceae such as E. coli, Citrobacter and Enterobacter spp. This group consists of ampicillin and amoxicillin. The susceptibility of Enterobacteriaceae is tested with an ampicillin disc (10 µg), that of staphylococci and streptococci with a penicillin disc (1 unit).
**β-lactamase inhibitor combinations**

These antimicrobial agents are combinations that include a penicillin-class antimicrobial agent and a second agent that has minimal antimicrobial activity, but functions as an inhibitor of most β-lactamases. In veterinary medicine, only clavulanic acid is currently used in some countries in combination therapies against mastitis.

The susceptibility of staphylococci to amoxicillin with clavulanic acid is tested with a cefoxitin disc (30 µg), and the susceptibility of *Enterobacteriaceae* with an amoxicillin/clavulanic acid disc (20/10 µg). Streptococci are not able to produce β-lactamase, therefore testing is not applicable.

**Cephalosporins**

The various representatives of cephalosporins can have a somewhat different activity against Gram-positive and Gram-negative bacteria. These agents are often referred to as 1st, 2nd, 3rd and 4th generation cephalosporins, based on the extent of their activity against the more antimicrobial-resistant, Gram-negative bacteria.

- **First (and second) generation cephalosporins** are active against Gram-positive streptococci and staphylococci, including the β-lactamase-producing staphylococci, but not against methicillin-(oxacillin) resistant staphylococcus (e.g. MRSA or methicillin-resistant *Staphylococcus aureus*). They are active against wild types of *Enterobacteriaceae*. This group includes cefalexin, cefaloni- um, cefalotin and cefapirin. **Cefalexin (30 µg) discs** are used to test the susceptibility of *Enterobacteriaceae*, cefoxitin discs (30 µg) are used for staphylococci and penicillin discs (1 unit) are used for streptococci.

- **Third and fourth generation cephalosporins** have a broad spectrum of activity both for Gram-positive and Gram-negative bacteria. They are generally resistant to a broad spectrum of β-lactamases, which makes them especially valuable for the treatment of Gram-negative infections. However, resistance occurs and their use should be carefully considered*. This group consists of ceftiofur, cefoperazone and cefquinome. The susceptibility of *Enterobacteriaceae* to these antimicrobials can be assessed with a cefotaxime disc (5 µg); however, the use of a disc with the specific therapeutic antimicrobial could result in different outcomes. The susceptibility of staphylococci is tested with a cefoxitin disc (30 µg) and streptococcal susceptibility is assessed with a penicillin disc (1 unit).

*Note: According to the WHO, 3rd and 4th generation cephalosporins are considered to be very critical antibiotics for human medicine and their use in veterinary medicine should therefore be carefully considered.
**Aminoglycosides**

*Aminoglycosides* are bactericidal antimicrobials with both Gram-positive and Gram-negative activity. Generally, this includes *Staphylococcus* spp. (including β-lactamase-producing species) and *Enterobacteriaceae*. *Streptococcus* spp. are naturally resistant to all molecules of this class.

This group of chemically-related drugs includes, among others, kanamycin, streptomycin, neomycin, gentamicin and spectinomycin. Framycetin is one of the compounds of neomycin; in fact, it is its strongest-acting compound. Aminoglycosides are affected in variable ways by bacterial inactivating enzymes, which results in differences in their spectrum of activity. For this reason, each aminoglycoside should be tested separately for a reliable evaluation of its efficacy.

Susceptibility is tested with a kanamycin disc (30 µg), a neomycin disc (10 µg), a gentamicin disc (10 µg) and an amikacin disc (30 µg).

**Lincosamides**

*Lincosamides* are moderate-spectrum antimicrobial drugs. These molecules inhibit protein synthesis and include lincomycin, clindamycin and pirlimycin. They are efficacious against most Gram-positive bacteria (staphylococci, streptococci, but not enterococci) and against Gram-positive and -negative anaerobe bacteria. These drugs are not active against most aerobic or facultative aerobic Gram-negative bacteria such as *Enterobacteriaceae*.

Susceptibility is tested with a clindamycin disc (2 µg) and inducible resistance is tested with a side-by-side test next to an erythromycin disc (15 µg) (see Special cases).

**Macrolides**

*Macrolides* are bacteriostatic agents that inhibit protein synthesis. This group includes erythromycin, gamithromycin, tilmicosin, tulathromycin and tylosin. They are active against staphylococci and streptococci although resistance occurs. The mastitis-causing, Gram-negative *Enterobacteriaceae* and *Pseudomonas* spp. are naturally resistant. Susceptibility is tested with an erythromycin disc (15 µg).

**Trimethoprim and Sulfonamides**

*Sulfonamides* are broad-spectrum antimicrobial agents usually combined with *trimethoprim* because of the synergy between both molecules. This combination affects different steps of the production of folic acid and thus that of purines, which are required for DNA synthesis. Their spectrum includes Gram-positive and Gram-negative bacteria and some protozoa. However, they are not active against anaerobe bacteria and at locations with tissue debris.

*Trimethoprim-sulfonamide combinations* (TMPS) are active against staphylococci, streptococci and *Enterobacteriaceae*.

Susceptibility is tested with a Trimethoprim-sulfamethoxazole disc (1.25-23.75 µg = 25 µg).

*Note:* According to the WHO, macrolides are considered to be very critical antibiotics for human medicine and their use in veterinary medicine should therefore be carefully considered.
**Tetracyclines**

Tetracyclines are bacteriostatic antimicrobials, which have a broad spectrum of activity against Gram-positive and Gram-negative bacteria. This group includes tetracycline, oxytetracycline, chlortetracycline and doxycycline and susceptibility is tested with a tetracycline disc (30 µg).

**Quinolones**

This group includes the older quinolones (e.g. nalidixic acid) and the newer fluoroquinolones, such as danofloxacin, enrofloxacinc, marbofloxacinc. All quinolones act by blocking DNA replication, and some quinolones also act on non-dividing bacteria. Especially the newer fluoroquinolones have a broad spectrum of activity against aerobic Gram-negatives and Gram-positives. They have a good activity against staphylococci and Enterobacteriaceae.

Susceptibility is tested with an enrofloxacinc disc (5 µg) or a ciprofloxacinc disc (5 µg).

**Combinations**

Several antimicrobial combinations have been approved for intramammary use. Especially for the combinations where a synergy has been proven against mastitis pathogens, the separate testing of molecules will underestimate sensitivity. Testing of the combination at ratios that are likely to be reached in the udder is therefore advised.

Cefalexin/kanamycin is an example of a combination with established synergy against *Staphylococcus aureus*, *Streptococcus uberis*, *Streptococcus dysgalactiae* and *Escherichia coli*. Susceptibility is tested with a cephalexin-kanamycin disc (C 15µg/K 30 µg).

*Note: According to the WHO, fluoroquinolones are considered to be very critical antibiotics for human medicine and their use in veterinary medicine should therefore be carefully considered.*

**Antimicrobial Susceptibility Testing on Milk Samples**

**Establishing antimicrobial panels**

Panels should be based on the availability of commercial pharmaceutical formulations. It is practical to prepare separate panels for Gram-positive and Gram-negative isolates.

**Gram-positives**

1. The **benzylpenicillin disc (1 unit)** is used to test the susceptibility of *Staphylococcus* spp. and *Streptococcus* spp. to penicillin (and penethamate). For these Gram-positives, penicillin can be also taken as a representative for moderate-spectrum penicillins such as ampicillin and amoxicillin. To evaluate β-lactamase production in staphylococci, not only the size but also the character of the edges of the diffusion zone should be taken into account (see Special cases).

2. The **cefoxitin disc (30 µg)** is used as a reliable test for the presence of the mecA gene in *Staphylococcus* spp. (MRSA). Staphylococci susceptible to cefoxitin are considered to be susceptible to narrow-spectrum, β-lactamase-resistant penicillins, to β-lactam/β-lactamase combinations (amoxicillin/clavulanic acid), and to all classes of cephalosporins. Cefoxitin-resistant strains (MRSA) are considered to be resistant to all β-lactams (see Special cases). In streptococci, oxacillin (1µ) needs to be tested separately from penicillin. Although streptococci do not produce β-lactamase, more cases are reported where streptococci are resistant to oxacillin despite penicillin sensitivity.

3. Aminoglycosides can be tested with a **neomycin disc (10 µg)** for *S. aureus* and *Enterobacteriaceae*, a **gentamicin disc (10 µg)** and a **kanamycin disc (30 µg)** for all bacterial species and an **amikacin disc (30 µg)** for staphylococci. None of these discs can predict resistance for the complete antibiotic class. The neomycin disc can be used to assess susceptibility to framycetin as well.

4. The **erythromycin disc (15 µg)** is used to test macrolide susceptibility in staphylococci and streptococci. This disc is always used next to a clindamycin disc to evaluate inducible clindamycin resistance (see Special cases).

5. The **clindamycin disc (2 µg)** is used to test the susceptibility of staphylococci and streptococci to pirlimycin and lincomycin. Inducible clindamycin resistance is tested with the D-test (see Special cases).

6. The **trimethoprim-sulfamethoxazole disc (25 µg)** is used to test the susceptibility of staphylococci and streptococci to trimethoprim-sulfona-mide combinations.

7. The **tetracycline disc (30 µg)** disc is used to test susceptibility of staphylococci and streptococci to this group of antimicrobials.

8. The **ciprofloxacin disc (5 µg)** or the **enrofloxacin disc (5 µg)** are used to test the susceptibility of staphylococci and streptococci to fluoroquinolones.

9. The **cefalexin/kanamycin disc (C 15 µg/K 30 µg)** is used to test the susceptibility of staphylococci and streptococci to Ubrolexin*. The **penicillin/framycetin disc (P 1 IU/F 100 µg)** is used to test the susceptibility of staphylococci and streptococci to Ubrostar*.
Gram-negatives

1. The ampicillin disc (10 µg) is used to test the susceptibility of *Enterobacteriaceae* to ampicillin and amoxicillin.

2. The amoxicillin-clavulanic acid disc (20 -10 µg) is used to test the susceptibility of *Enterobacteriaceae* to amoxicillin-clavulanic acid combinations. Note: if Gram-negative bacteria test resistant to the combination amoxicillin-clavulanic acid, they should also be reported as resistant to ampicillin (see Special cases).

3. The cefalexin (30 µg) is used to test *Enterobacteriaceae* to first and second generation cephalosporins such as cefalexin and cefapirin.

4. The cefotaxime disc (5 µg) is used to test for resistance to all β-lactams. *Enterobacteriaceae* resistant to cefotaxime are considered to be clinically resistant to all penicillins and cephalosporins and β-lactam inhibitor combinations, even though the diameters for these antimicrobials indicate susceptibility (see Special cases).

5. Aminoglycoside susceptibility can be tested with a neomycin disc (10 µg), a gentamicin disc (10 µg), a kanamycin disc (30 µg) and an amikacin disc (30 µg) for the specific susceptibility of *Enterobacteriaceae* to each antimicrobial separately.

6. The trimethoprim-sulfamethoxazole disc (25 µg) is used to test the susceptibility of *Enterobacteriaceae* to trimethoprim-sulfonamide combinations.

7. The tetracycline disc (30 µg) disc is used to test susceptibility of *Enterobacteriaceae* to tetracyclines separately.

8. The ciprofloxacin disc (5 µg) or enrofloxacin disc (5 µg) is used for testing the susceptibility of *Enterobacteriaceae* to fluoroquinolones.

9. The cefalexin/kanamycin disc (C 15 µg/K 30 µg) is used for testing the susceptibility of *E.coli* to Ubrolexin®. The penicillin/framycetin disc (P 1 IU/F 100 µg) is used to test the susceptibility of *E. coli* to Ubrostar®.

Selective reporting

Translating reference molecules to commercial preparations will enhance the practical use of the lab results. An example of a therapeutic guide for mastitis pathogens and antimicrobials is given in addendum 1.

Enterococcal infections in bovine mastitis occur; nevertheless, they are not widespread. During bacterial isolation, they are often misidentified as streptococci. Enterococci are resistant (natural and acquired resistance) to various antibiotics.

- Susceptibility to penicillins in enterococci can be tested with an ampicillin disc (2µg). Enterococci resistant to ampicillin can be considered resistant to all penicillins including amoxicillin and amoxicillin with clavulanic acid.

- All enterococci are naturally resistant to cephalosporins, *in vitro* outcomes should be disregarded.

- All enterococci are naturally resistant to aminoglycosides, although synergy with β-lactams may occur.

- Resistance against macrolides amongst enterococci is widespread.

- Quinolones are generally only moderately active against enterococci. Several genetic mutations can cause resistance to quinolones in enterococci.