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Penicillin susceptibility of *Streptococcus sp*. isolated from Austrian dairy cows.

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submitted by
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DANKE

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1. Introduction

Mastitis is known as the most frequent and costliest disease in dairy cattle (Hillerton & Berry, 2005; Petersson-Wolfe, Leslie, & Swartz, 2018). The disease is mostly detected by clinical signs, such as abnormalities in milk and udder, and moreover has an impact in animal production and welfare (Hillerton & Berry, 2005). A number of factors, such as milk production losses, drugs, discarded milk, veterinary services, labor, product quality, material and investments, diagnostics, other diseases and culling, have an impact on the costs (Halasa, Huijps, Østerås, & Hogeveen, 2007). The final costs for clinical mastitis per cow per year vary from 17 to 261€ (Doehring & Sundrum, 2019). The relative costs differ between countries and regions, but the economic principles are the same (Halasa et al., 2007). The loss of milk quantity is affected by the pathogenic species and the mastitis form (Winter, 2008). Therefor the correct determination of these two factors is essential.

1.1. Mastitis

Mastitis is an inflammatory disease of the udder, usually caused by bacteria (Hillerton & Berry, 2005), but can also have a noninfectious etiology (Bradley, 2002). The disease can be segmented in latent, subclinical and clinical forms (Winter, 2008). Regarding the disease process it can be distinguished between acute and chronic mastitis (Winter, 2008). More than 130 micro-organism have been reported to cause mastitis, the most common ones are *Staphylococcus (S.) aureus*, *Streptococcus (S.) uberis*, *Streptococcus (S.) dysgalactiae*, *Streptococcus (S.) agalactiae* and various Gram-negative bacteria (WATTS, 1988). Mastitis pathogens can be divided in contagious (*S. aureus, S. dysgalactiae and S. agalactiae*) and environmental (*Enterobacteriacae* and *S. uberis*) (Bradley, 2002). Different symptoms can be seen as a result of the different mastitis forms. While animals, suffering from clinical mastitis, show fatal symptoms like a swollen, painful udder of higher temperature, subclinical mastitis forms mainly manifest with an increased somatic cell count (SCC) (Winter, 2008).

There are economic losses, consisting of costs for treatment, production losses, culling, changes in product quality and the risk of other diseases (Halasa et al., 2007).

1.2. Streptococci

Streptococci, belonging to the lactic acid group of bacteria, are Gram-positive, spherical and catalase negative organisms, many of them facultative anaerobes (Póntigo, Moraga, & Flores, 2015). By now more than 60 *Streptococcus* species have been described (Haenni, Lupo, & Madec, 2018). A classification can be made according to the hemolysis behavior, whereby alpha-hemolytic (viridans Streptococci), beta-hemolytic (complete hemolysis) and gamma hemolytic (absent hemolysis) Streptococci can be divided (Gatermann & Miksits, 2009). Further beta-hemolytic Streptococci are divided into serogroups (A-H, K-V) by the Lancefield schemata (Gatermann & Miksits, 2009). This classification is based on the antigenic reaction of the cell wall-associated carbohydrates, whereby several antibodies can react with isolates of the same species (Haenni et al., 2018). Not only some of the most important human pathogens, such as *S. pyogenes* and *S. pneumoniae*, belong to the *Streptococcus* genus, but also very important veterinary ones, *S. agalactiae*, *S. dysgalactiae*, *S. equi*, *S. uberis* (Aaarestrup &

Schwarz, 2006). The three most commonly species isolated from bovine mammary glands are *S. agalactiae*, *S. dysgalactiae* and *S. uberis* (Hillerton & Berry, 2005; WATTS, 1988). However the incidence of *S. agalactiae* has decreased in the past 20 years and is now rarely isolated from cattle mastitis, which is attributable to hygiene measures and guidelines for good practices (Haenni et al., 2018).

1.2.1. *Streptococcus uberis*

S. uberis belongs to the beta-hemolytic Streptococci, Lancefield-serogroup E (Seelemann, 1948). However, Winter (2008) whereas describes the species with mostly no belonging to the Lancefield groups and an uncomplete ore complete hemolysis. Also Haenni et al. (2018) classify *S. uberis* to the non-Lancefield group. On blood agar the bacteria shows small, transparent, mucoid colonies (Winter, 2008). The bacteria is one of the common causes of mastitis, next to *S. aureus*, *S. dysgalactiae* and *S. agalactiae* (Haenni et al., 2018; Hillerton & Berry, 2005) and is present in 15 to 35 % of all cases (Aaarestrup & Schwarz, 2006; Esener et al. 2018). As an environmental associated pathogen, *S. uberis* can be found in every stable and is spread among milking times (Winter, 2008). McDougall et al. (2020) stated that the pathogen can manifest in contagious and environmental form. Although *S. uberis* in general has a slight pathogenic potential, in the case of bad immune defense against infection it can cause subclinical or acute catarrhal mastitis with fever and changes in milk appearance (Winter, 2008). The pathogen is known to have a susceptibility against Penicillin (McDougall et al., 2020).

1.2.2. *Streptococcus dysgalactiae*

S. dysgalactiae is mostly allocated to Lancefield group C (Winter, 2008) and G (Haenni et al., 2018) and shows small colonies and greening hemolysis on blood agar (Winter, 2008). It includes two subspecies, *S. dysgalactiae* subsp. *dysgalactiae* and *S. dysgalactiae* subsp. *equisimilis* (Aaarestrup & Schwarz, 2006). The pathogen can be found in infected udder as well as in the environment and thus can be spread during milking and between milking times

(Winter, 2008). Together with *S. uberis, S. agalactiae, S. aureus* and *Escherichia* (*E.*) *coli* it accounts for almost 80 % of the mastitis diagnosis (Bradley, 2002). Infected animals usually suffer from subclinical mastitis, but also acute catarrhal mastitis with flakes in the milk are possible (Winter, 2008).

1.2.3. Treatment

Purposes of mastitis therapy not only include curing of the individual animal, but also decreasing infection pressure in herd and improving milk quality (Winter, 2008).

The conventional treatment of mastitis in lactating cows is antibiotic therapy, by intramammary inoculation of an antibiotic-containing paste or intramuscular injection (Hillerton & Berry, 2005). Doehring & Sundrum (2019) reported a use of last resort substances in 32% of mastitis cases. Though antibiotic application to eliminate or reduce bacteria is only a part of mastitis therapy (Winter, 2008). Reducing the transmission of mastitis pathogens, for instance, by improving hygiene, is economically reasonable (Gussmann et al., 2018).

A correct determination of the pathogen as well as its resistance behavior are required for the use of antibiotics and spectrum efficacy, mode of action, therapeutic index and tissue penetration are relevant criteria to choose an antibiotic agent (Winter, 2008). In Germany β-lactam antibiotics are the most common used for the treatment of mastitis (Schwarz, Kadlec, & Silley, 2013). The first choice antibiotic to use for mastitis caused by *S. uberis* and *S. dysgalactiae* is penicillin, especially benzylpenicillin, but also cloxacillin, penethamathydrojodid, ampicillin and lincosamides (pirlimycin, lincomycin), macrolides (tylosin), as well as cephalosporin (cefquinom) are used alternatively in various schemata (Winter, 2008).

While intramammary preparations often include more than one antibiotic and moreover corticosteroid to treat inflammation, intramuscular products are usually single active, often benzylpenicillin (Hillerton & Berry, 2005).

Dairy cows should spend a nonlactating phase, six to eight weeks, prior to calving (Hillerton & Berry, 2005). During this period intramammary inflammation can be cured, but also persist or new infections may occur (Wittek, Tichy, Grassauer, & Egger-Danner, 2017). Therefor there

have been trials for dry cow treatment with antibiotics, which showed a prophylactic benefit of 82% in the rate of new intramammary infection than achieved in lactation (Hillerton & Berry, 2005). As any use of antibiotic, antibiotic dry-off treatment, can result in resistance and thus should be considered critically (Wittek et al., 2017).

Also associated symptoms have to be treated. Frequent and complete milking of lactating cows eventually in combination with Oxytocin supports the elimination of contagious pathogens and their toxins (Winter, 2008). Moreover, as severe and moderate clinical mastitis forms are related with pain, non-steroidal anti-inflammatory drugs (NSAIDs) are useful to improve animal welfare with an anti-inflammatory, antipyretic and analgesic impact (Petersson-Wolfe et al., 2018). Besides the medical treatment a proactive management, including teat cleaning, stable hygiene, teat condition, species appropriate keeping and correct drying off is essential (Winter, 2008).

Table 1: Examples for therapy schemata for *Streptococcus* infections (Winter, 2008)

Agent	Dose	Duration	Application	Pathogen	Combination/Date
Benzyl-penicillin	3 mio. IU	3 d, every 12 or 24 h	IMM	Streptococci	ev. combination with parenteral therapy
	0.3 g/quarter	5 d, every 24h	IMM	*S. uberis*	-
	9.5 mg/kg	5d, every 12 h	par	*S. uberis*	combination with Benzylpenicillin IMM
	1 mio. IU	1x	IMM	Streptococci	drying off
Ampicillin	10 mg/kg	3-5 d, every 12 h	par	*S. uberis, S. dysgalactiae*	combination with IMM therapy
Cloxacillin	500-100 mg	1x	IMM	Streptococci	drying off
Penethamat-hydrojodid	5 mio IU	3 d, every 24 h	par	*S. uberis, S. dysgalactiae*	drying off, peripartum, single use or combination with IMM therapy
Tylosin	5-10 mg/kg	3-5 d, every 12 h	par	*S. uberis*	drying off, peripartum, combination with IMM therapy
Pirilimycin	50 mg/quarter	8 d	IMM	*S. uberis*	ev. Combination with parenteral therapy

Lincomycin	300 mg/quarter	2-3 d, every 12 h	IMM	*S. uberis*	ev. combination with parenteral therapy
Cefquinom	75 mg/quarter	2 d, every 12 h	IMM	*S. uberis, S. dysgalactiae*	-

IMM = intramammary; par = parenteral

1.3. Antimicrobial resistance

Antimicrobial resistance is defined as a gradually variable non-susceptibility of bacteria to antimicrobial agents (Schwarz et al., 2013). Antibiotic resistance has been recognized as a global health problem for many decades (Marshall & Levy, 2011) in human as well as in veterinary medicine (Rose et al., 2018). Any use of antimicrobials results in a selective pressure, which is why pathogenic as well as non-pathogenic commensal bacteria can develop resistance to the respective agent (Guardabassi et al., 2015). The increase of bacteria resistant to antibiotic is widely attributed to indiscriminate antimicrobial use (Ekakoro & Okafor, 2019). Therefor monitoring of antimicrobial resistance is on many national and international agendas (Rose et al., 2018).

There are two types of resistance that can be differentiated. The species- or genus specific intrinsic resistance is based on absence or inaccessibility of the target site of the antimicrobial agent (Schwarz et al., 2013). As it occurs in bacteria, that have never been susceptible for the drug it should be referred to as insensitivity (Guardabassi & Courvalin, 2006). The strain specific acquired resistance, due to resistance mediating mutations (Schwarz et al., 2013) is the greater danger to animal and human health because normally susceptible bacteria populations develop to resistant forms and thus may lead to therapeutic failure (Guardabassi & Courvalin, 2006). Regardless of the resistance-type, a number of different mechanisms of resistance exist (Schroeder, Brooks, & Brooks, 2017).

1.3.1. Antimicrobial resistance and streptococci

There are several national surveillance and monitoring programs in veterinary medicine across Europe, but streptococci of animal origin were poorly included, wherefore data on their resistance to antimicrobials is limited (Haenni et al., 2018).

In general all streptococcal species show a high susceptibility to penicillin, which is the most common used drug to control these bacteria (McDougall et al., 2020). β-lactam resistance in veterinary medicine has been documented is *S. uberis*, *S. dysgalactiae* and *S. agalactiae*, whereas most studies report the absence of benzylpenicillin resistance (Haenni et al., 2018).

S. uberis and *S. dysgalactiae* are also known as susceptible to chloramphenicol (Aaarestrup & Schwarz, 2006). However antibiotic resistance against aminoglycosides, macrolides, lincosamides and tetracycline has been detected (Haenni et al., 2018; Schwarz et al., 2013; Šlosárková et al., 2019). Additionally (Rose et al., 2018) reported antimicrobial resistance of *S. uberis* and *S. dysgalactiae* against erythromycin and tetracycline.

1.3.2. Detection of antimicrobial resistance

Haenni et al. (2018) mentions two methods as the most frequently used to determine antimicrobial resistance: antibiograms performed by disc diffusion and MIC performed by broth microdilution. The qualitative or quantitave results of these techniques are interpreted according to official guidelines (EUCAST, CLSI, Antibiogram Committee of French Microbiologiy Society (CA-SFM) and the studied isolates can be classified as susceptible, intermediate or resistant to the tested antibiotic. Moreover the large-scale genomic analyses for the detection of resistance mechanisms and capsular types, for the sequence-based prediction of beta lactam resistance using the penicillin-binding protein (PBP) transpeptidase signatures, and for the prediction of antimicrobial profile and its potential evolution toward resistance over time is used recently (Haenni et al., 2018).

1.3.3. β-lactam resistance

Including penicillin, cephalosporin, carbapenem and monobactam, β-lactams belong to the largest classes of antibiotics (Haenni et al., 2018; Worthington & Melander, 2014). Their antibiotic effect is based on the imitation of the D-Ala-D-Ala dipeptide of PBPs (Zapun et al., 2008). PBPs are cell wall transpeptidases, catalyzing the assembly of cell wall (McDougall et al., 2020). By forming an acyl-enzyme complex with PBPs, β-lactams inhibit their transpeptidation activity and destroy the integrity of the cell wall, leading to cell lysis (Worthington & Melander, 2014).

In general there are two mechanism which can lead to resistance to β-lactam antibiotics: production of β-lactamases and the production of modified PBPs with a lower affinity to β-lactam antibiotics (Worthington & Melander, 2014). The expression of the enzyme β-lactamases, which is the most common resistance-mechanism in bacteria, leads to the hydrolytic cleavage of the β-lactam moiety of the drug and therefor to inefficiency (Kotra & Mobashery, 1998). β-lactam resistance due to modified PBPs is a result of changes within the three amino acid motifs SXXK, SXN, and KT(S)G of the transpeptidase (McDougall et al., 2020).

Streptococci are incapable of acquiring exogenous β-lactam resistance genes and are not carrying β-lactamase, but are able to progressively mutate their own PBPs, whereby resistance is achieved by modifying class B PBP2B and PBP2X (Haenni et al., 2018)

2. Aim

This diploma thesis was done within the scope of the project Advancement of Dairying in Austria (ADDA; Area 2-Workpackage 2). Prior in this project mastitis pathogens were isolated from 110 Austrian dairy farms.

The aim of this thesis was to determine the antibiotic resistance of 241 *Streptococcus* isolates against benzylpenicillin. The *Streptococcus* isolates included *S. uberis, S. dysgalactiae, S. pluranimalium, S. parauberis, S. macedonicus, S. pasteurianus, S. equi, S. equinus, S. lutetiensis and S. salivarius.*

The results of this thesis can be used as an orientation referring to the incidence of *Streptococcus spp*. in Austria. A correct determination of the species is essential to detect antibiotic resistance and as a consequence be able to make a decision for the ideal treatment.

3. Material and Method

3.1. Samples

241 Streptococci isolates of ten different *Streptococcus species* (Table 2) of Austrian dairy cows were collected from veterinarians and farmers between 1st October 2015 and 29th September 2016. 110 farms and 231 animals were included. Data of these isolates were collected as part of the project "ADDA – Advancement of Dairying in Austria"(Firth et al., 2017). The farmers were actively recruited by enrolled veterinarians involved in the ADDA project (Firth et al., 2017). Bovine quarter milk samples were taken from diseased and healthy animals and included clinical or subclinical mastitis cases as well as routine checks (Schabauer et al., 2018). General information (ear tag number/animal ID, farm ID, person who collected the sample), as well as information on animal health and mastitis severity was recorded (Schabauer et al., 2018). After the first bacteriological analyses in participating Austrian veterinary laboratories (n=5) the isolated cultures were sent as cryogenic vials to the Institute if Milk Hygiene at the University of Veterinary Medicine in Vienna (Schabauer et al., 2018).

Table 2: Streptococci species

Species	Number of isolates (n)	Number of isolates (%)
S. uberis	156	64.7
S. dysgalactiae	70	29.0
S. pluranimalium	4	1.7
S. parauberis	3	1.2
S. macedonicus	2	0.8
S. pasteurianus	2	0.8
S. equi	1	0.4
S. equinus	1	0.4
S. lutetiensis	1	0.4
S. salivarius	1	0.4
Total	**241**	**100.0**

3.2. Preparation of inoculum

A 10µl inoculation loop was used to take material from cryocultures. Bacteria were inoculated in 1ml BHI-Y and incubated for 6-8 hours at 37°C. After that each isolate was streaked on a non-selective Columbia-Agar with 5% sheep blood (COS, Biomeriox France) with a 1 µl inoculation loop. One agar plate was used for 8 Streptococci isolates. The plates were incubated for 24 hours at 37°C.

3.3. Agar diffusion test

The agar diffusion test was performed by the EUCAST principles (antimicrobial susceptibility testing; EUCAST disk diffusion method; Version 5.0, January 2015) and the clinical breakpoints (Breakpoint tables for interpretation of MICs and zone diameters, Version 6.0, January 2016) (Table 3) were used to determine antibiotic resistance.

A colony from the non-selective COS agar plates was taken with a sterile cotton swab and suspended in 3ml 0.9% sodium chloride (NaCl) in a tubule (tubule with round bottom, polystyrol 5ml with closing cab). The suspensions was standardized to the density of a McFarland 0.5 standard, which corresponds to a nucleus density of 1-2 x10^8. The density of the suspension was checked in a spectrophotometer (UV spectrophotometer UV-1800, Shimadzu, Kyoto, Japan) with a 1 cm light path and matched cuvettes. The absorbance at 625nm should be in the range of 0.07-0.13.

Within 15 minutes of preparation the adjusted inoculum suspensions were streaked on a Müller Hington agar with 5% mechanically defibrinated horse blood and 20mg/L β-NAD (MH-F, Biomerioux, France). Therefor a sterile cotton swab was dipped into the suspension and the excess fluid was removed by turning the swab against the inside of the tubule. Then the inoculum was spreaded over the entire surface of the plate by swabbing in three directions at an interval of 90°. A benzylpenicillin flake was positioned in the middle of each plate and after an incubation for 18 ± 2 hours the zone of inhibition (in mm) was measured.

Table 3: Breakpoint

Antibiotic	Disk content	Zone Diameter breakpoint (mm)	
benzylpenicillin	1 unit	≥ 18 = S	< 18 = R

S: susceptible; R: resistant

4. Results

In total 241 Streptococcus sp. were investigated in this study comprising of 156 *S. uberis*, 70 *S. dysgalactiae*, 4 *S. pluranimalium,* 3 *S. parauberis,* 2 *S. macedonicus,* 2 *S. pasteurianus,* 1 *S. equi*, 1 *S. equinus*, 1 *S. lutetitiensis* and 1 *S. salivarius* isolates.

4.1. Background information of samples

Altogether 110 farms and 231 animals were included. Some farms as well as animals have been sampled more than one time. The number of different farms and cows and also the times they have been sampled was categorized for *S. uberis*, *S. dysgalactiae* and other *Streptococcus* species (Table 4).

99 of the 156 *S. uberis* samples were taken by farmers, 42 from veterinarians and for 15 there was no information about the sampler. From 70 *S. dysgalactiae* isolates 38 were sampled by farmers, 26 by veterinarians and for six there was no information. 11 samples of the other 15 *Streptococcus* species were taken by farmers, the other four from veterinarians.

Table 4: Number of farms and cows

Species	***S. uberis* (n)**	***S. dysgalactiae* (n)**	**Other Streptococcus species (n)**
Farms total	**82**	**52**	**13**
Times sampled			11
1	50	38	2
2	14	10	-
3	10	4	-
4	2	-	-
5	4	-	-
6	1	-	-
14	1	-	-
Cows total	**152**	**70**	**15**
Times sampled			
1	149	70	15
2	3	-	-

The samples were taken from diseased and healthy animals and thus included clinical and subclinical mastitis cases and routine checks (before drying off). Moreover an assessment of the milk samples with the California mastitis test (CMT) was done. The CMT gives a first lead to mastitis by oblique somatic cell count determination. The reason for sampling as well as the different scores of the CMT of *S. uberis*, *S. dysgalactiae* and the other *Streptococcus* species samples were evaluated (Table 5, 6, 7).

Table 5: Reason for sampling of *S. uberis* isolates

Reason for sampling	**Number of isolates (n)**	**Number of isolates (%)**
Acute mastitis	78	50.00
Chronic mastitis	-	-
Changes in milk appearance	5	3.21
Increased SCC [1]	43	27.56
Control BDO [2]	13	8.33
Increased SCC plus control BDO	2	1.28
Unknown [3]	13	8.33
Others	2	1.28
CMT score		
Very high	66	42.31
High	14	8.97
Low	9	5.77
Negative	13	8.33
Unknown	54	34.62
Total	**156**	**100.00**

[1] SCC= somatic cell count according to CMT (California mastitis test).

[2] BDO = before drying off.

[3] No information provided on accompanying documentation.

Table 6: Reason for sampling of *S. dysgalactiae* isolates

Reason for sampling	Number of isolates (n)	Number of isolates (%)
Acute mastitis	33	47.14
Chronic mastitis	1	1.43
Changes in milk appearance	5	7.14
Increased SCC [1]	14	20.00
Control BDO [2]	3	4.29
Increased SCC plus control BDO	-	-
Unknown [3]	11	15.71
Others	3	4.29
CMT score		
Very high	30	42.86
High	7	10.00
Low	4	5.71
Negative	1	1.43
Unknown	28	40.00
Total	**70**	**100.00**

Table 7: Reason for sampling of other Streptococcus isolates (*S. plurinarium, S. parauberis, S. macedonicus, S. pasteurianus, S. equi, S. equinus, S. lutetiensis, S. salivarius*)

Reason for sampling	Number of isolates (n)	Number of isolates (%)
Acute mastitis	3	20.00
Chronic mastitis	-	-
Changes in milk appearance	-	-
Increased SCC [1]	4	26.67
Control BDO [2]	2	13.33
Increased SCC plus control BDO	2	13.33
Unknown [3]	2	13.33
Others	2	13.33
CMT score		
Very high	4	26.67
High	5	33.33

Low	1	6.67
Negative	1	6.67
Unknown	4	26.67
Total	**15**	**100.00**

4.2. Benzylpenicillin resistance

In total eight *Streptococcus* isolates were resistant to benzylpenicillin, corresponding of 3.32% (Figure 1). Antibiotic resistance could only be determined for *S. uberis* and *S. dysgalactiae* isolates. All of the other strains showed susceptibility for benzylpenicillin.

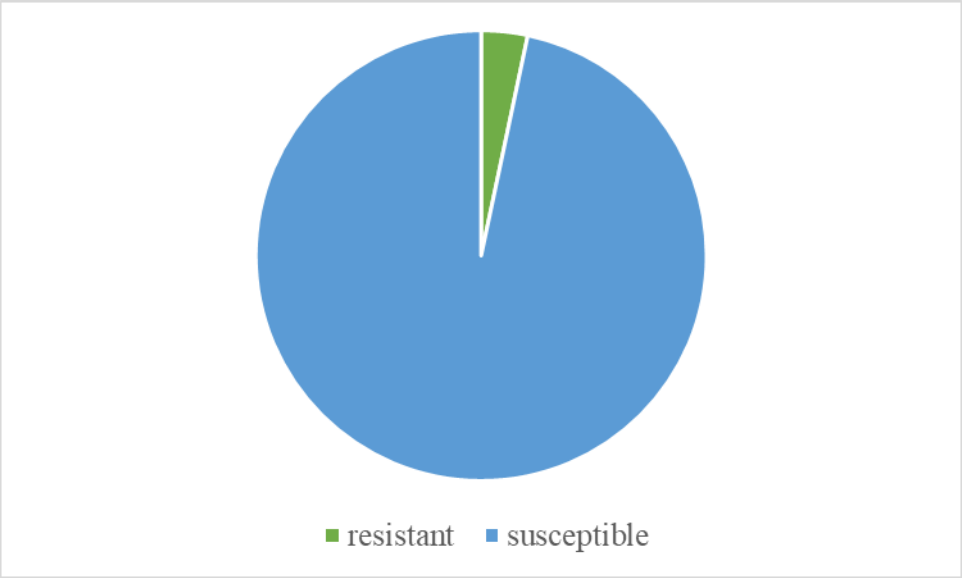

Figure 1: Benzylpenicillin resistance & susceptibility of 241 Streptococcus spp.

Six of the tested *Streptococcus uberis* strains showed resistance against benzylpenicillin, which correlates a percentage of 3.85% (Figure 2). This six isolates were all sampled at different farms and from different cows. Two were taken by a farmer, two by a veterinarian and for two there is no information about the sampler. Three of these resistant *S. uberis* samples were taken due to acute mastitis cases, one was a control sample before drying of (BDO).For the others there is no information. For two samples taken because of acute mastitis the CMT resulted in the score "very high", for one no information is provided.

Figure 2: Benzylpenicillin resistance & susceptibility of 156 *S. uberis* isolates

Two of the tested *S. dysgalactiae* strains showed resistance against benzylpenicillin, which correlates a percentage of 2.86% (Figure 3). Both were sampled at different farms and from different cows, whereby one was taken because of an increased somatic cell count (SCC) by a farmer and the other because of an acute mastitis case from a veterinarian. The sample taken because of an acute mastitis had a low CMT score, whereas the other sample had a high CMT score.

Figure 3: Benzylpenicillin resistance and susceptibility of 70 *S. dysgalactiae* isolates

5. Discussion

This diploma thesis was done within the scope of the project Advancement of Dairying in Austria (ADDA; Area 2-Workpackage 2). This project determined streptococci as one of the most common bacteria in bovine mastitis next to staphylococci and Enterobacteriacae.

For this thesis data from 231 cows on 110 Austrian dairy farms were consulted. Isolates were taken by veterinarians and actively recruited farmers. These were not randomized samples throughout Austria, but a convenience sample within an observational study (Firth et al., 2017). Further there were no restrictions for farm size, production system, free-stalls or tie-stalls, alpine or valley farms (Firth et al., 2017).

For species identification, species-specific PCR was used for *S. uberis* and *S. dysgalactiae* and 16S rRNA gene and 18S rRNA gene sequencing was used for the other streptococci, after DNA isolation performed according to the Chelex extraction protocol (Schabauer et al., 2018).

In total 241 *Streptococcus sp*. were investigated. 64.73% of the isolates were detected as *S. uberis*, 29.00% as *S. dysgalactiae* and the other 6.27 % included the species *S. pluranimalium*, *S. parauberis*, *S. macedonicus*, *S. pasteurianus*, *S. equi*, *S. equinus*, *S. lutetiensis*, *S. salivarus*. These results correspond the statements of current literature of *S. uberis* and *S. dysgalactiae* being the most isolated streptococci in bovine mastitis (Haenni et al., 2018; Hillerton & Berry, 2005; WATTS, 1988; Winter, 2008).

The samples were taken from diseased and healthy animals, including clinical and subclinical mastitis cases and routine checks, and CMT was made. A significant association between the mastitis severity, as well as CMT score and the present *Streptococcus* species could be detected. 50.00% of the *S. uberis* samples were taken because of acute mastitis cases, for *S. dysgalactiae* 47.14% were acute mastitis cases. Only 20% of the other *Streptococcus* species were taken because of acute mastitis, 26.67% of them were taken because of an increased SCC. 42.31% of the milk samples containing *S. uberis* and 47.14% of those containing *S. dysgalactiae* had the CMT score “very high”. For the other species it was 33.33% with the CMT score “high” and 26.67% with the score “very high”. However it has to be mentioned that for many samples no information about the CMT score was provided.

The sampler and also factors relevant to the animal, such as appetite and consciousness, were not detected as relevant factors in the statistical model.

3.32% of all tested *Streptococcus* isolates were detected as resistant to benzylpenicillin, including only isolates of *S. uberis* and *S. dysgalactiae.* Resistance to benzylpenicillin could be determined for 3.85% of the *S. uberis* and 2.86% of the *S. dysgalactiae* strains.

(Rose et al., 2018) did not detect any resistance of *S. uberis* to benzylpenicillin, though 35.6% were determined as intermediate. The agar diffusion test in this thesis was performed by the EUCAST principles (antimicrobial susceptibility testing; EUCAST disk diffusion method; Version 5.0, January 2015) and the clinical breakpoints (Breakpoint tables for interpretation of MICs and zone diameters, Version 6.0, January 2016) were used to determine antibiotic resistance, where only a distinction is made between susceptible and resistant. (Rose et al., 2018) further recorded a susceptibility of *S. dysgalactiae* against benzylpenicillin of 100%. Countries included in this study were Belgium, Czech Republic, Denmark, France, Germany, Italy, the Netherlands, Spain and the United Kingdome.

Šlosárková et al. (2019) also did not detect resistance of *S. uberis* against benzylpenicillin in Czech dairy herds and detected 35.0% as intermediate. All of the *S. dysgalactiae* isolates were susceptible to benzylpenicillin (Šlosárková et al., 2019).

In Germany no resistance of *S. uberis* or *S. dysgalactiae* against penicillin could be established in 2013 (Schwarz et al., 2013).

Even though several studies across Europe report the absence of resistance to benzylpenicillin in *S. uberis* and *S. dysgalactiae*, a slow but clear shift of strains from full toward decreased susceptibility can be recognized (Haenni et al., 2018).

The mechanism leading to β-lactam antibiotic resistance can either be the production of β-lactamases or the production of modified PBPs with a lower affinity to β-lactam antibiotics (Worthington & Melander, 2014).

However, resistance against other antibiotics have been reported. Studies including antimicrobial susceptibility testing to macrolides report from resistance of *S. uberis* to erythromycin between 12,5% and 60% and of *S. dysgalactiae* between 11,4% and 100%

(Haenni et al., 2018; Rose et al., 2018; Schwarz et al., 2013). Rose et al. (2018) detected an antimicrobial resistance to tylosin of *S. uberis* of 20.2%.

Moreover there a reports about antimicrobial resistance of *S. uberis* and *S. dysgalactiae* to lincosamides between 0% and 42.9% (Haenni et al., 2018; Schwarz et al., 2013; Šlosárková et al., 2019) and to aminoglycosides between 28% and 67.1% (Schwarz et al., 2013; Šlosárková et al., 2019)

The highest percentage of resistance in streptococci can mostly be found to tetracycline. *S. uberis* is reported to have a resistance to tetracycline between 36.7% and 63.2% (Haenni et al., 2018; Rose et al., 2018; Schwarz et al., 2013; Šlosárková et al., 2019). The resistance of *S. dysgalactiae* to tetracycline is between 56.8% and 66.5% (Rose et al., 2018; Schwarz et al., 2013; Šlosárková et al., 2019). Haenni et al. (2018) reports a very variable antimicrobial resistance of *S. uberis* and *S. dysgalactiae* between 1.8% and 100%.

No resistance of *S. uberis* and *S. dysgalactiae* to cephalosporin has been reported (Rose et al., 2018; Schwarz et al., 2013).

However, due to the fact that benzylpenicillin is the most common used drug for treating clinical mastitis, the knowledge about the resistance of mastitis pathogens against this antibiotic is important. The assumption, of former and current literature, of a high susceptibility of streptococci, to benzylpenicillin could be reconfirmed in this thesis. Nevertheless it must be mentioned that several studies across Europe determine a shift of streptococci strains from full towards decreased susceptibility to benzylpenicillin (Haenni et al., 2018). This fact and the resistances to other antimicrobials mentioned before, again alert the importance of responsible handling of antimicrobials. Even though benzylpenicillin can still be recommended as first line therapy for bovine mastitis caused by streptococci, ongoing monitoring of antimicrobial resistances is important.

6. Summary

This diploma thesis was done within the scope of the project Advancement of Dairying in Austria (ADDA; Area 2-Workpackage 2). Prior in this project mastitis pathogens were isolated from 110 Austrian dairy farms.

The aim of this thesis was to determine the antibiotic resistance of 241 *Streptococcus* isolates against benzylpenicillin.

64.73% of the isolates were detected as *S. uberis*, 29.00% as *S. dysgalactiae* and the other 6.27 % included the species *S. pluranimalium*, *S. parauberis*, *S. macedonicus*, *S. pasteurianus*, *S. equi*, *S. equinus*, *S. lutetiensis*, *S. salivarus*.

The present *Streptococcus* species have been compared to the mastitis severity as well as the CMT score, where a significant association could be detected. While 50.00% of the *S. uberis* and 47.14% of the *S. dysgalactiae* samples were taken because of acute mastitis, only 20% of the other *Streptococcus* species could be associated with acute mastitis. The highest percentage of *S. uberis* (42.31%) and *S. dysgalactiae* (47.14%) had the CMT score “very high”. 33.33% of the other species had the CMT score “high” and 26.67% “very high.

An agar diffusion test of all samples was done to test the resistance to benzylpenicillin. 3.85% of the 156 *S. uberis* and 2.86% of the 70 *S. dysgalactiae* strains have been detected as resistant to benzylpenicillin. No resistance could be found in any other species.

Summarizing the recommendation by former literature, of benzylpenicillin as first line therapy for bovine mastitis caused by streptococci, can be confirmed. Nevertheless the importance of responsible handling of antimicrobials must be mentioned.

7. Zusammenfassung

Diese Diplomarbeit wurde im Rahmen des Projekts Advancement of Dairying in Austria (ADDA; Area 2-Workpackage 2) erstellt. Zuvor wurden im Rahmen dieses Projektes Mastitis Pathogene von 110 österreichischen Milchviehbetrieben isoliert.

Ziel dieser Arbeit war es, die Antibiotikaresistenz gegen Benzylpenizillin von 241 *Streptokokkus*-Arten zu bestimmen.

64,73% der Isolate wurden als *S. uberis*, 29,00% als *S. dysgalactiae* und die anderen 6,27% als *S. pluranimalium*, *S. parauberis*, *S. macedonicus*, *S. pasteurianus*, *S. equi*, *S. equinus*, *S. lutetiensis*, *S. salivarus* identifiziert.

Die vorliegenden *Streptokokkus*-Arten wurden mit dem Schweregrad der Mastitis sowie dem CMT-Score verglichen, wobei ein signifikanter Zusammenhang festgestellt werden konnte. Während 50,00% der *S. uberis*- und 47,14% der *S. dysgalactiae*-Proben wegen akuter Mastitis entnommen wurden, konnten nur 20% der anderen Streptokokkus-Arten mit akuter Mastitis in Verbindung gebracht werden. Der höchste Prozentsatz von *S. uberis* (42.31%) und *S. dysgalactiae* (47,14%) hatte den CMT-Wert „sehr hoch“. 33,33% der anderen Arten hatten einen CMT-Wert von „hoch“ und 26,67% von „sehr hoch“.

Ein Agardiffusionstest aller Proben wurde durchgeführt, um die Empfindlichkeit gegenüber Benzylpenicillin zu überprüfen. Bei 3,85% der 156 *S. uberis*- und 2,86% der 70 *S. dysgalactiae*-Stämmen wurde Resistenz gegen Benzylpenicillin nachgewiesen. Bei keiner anderen Art konnte eine Resistenz gefunden werden.

Zusammenfassend kann die Empfehlung, von Benzylpenicillin als Erstlinientherapie für durch Streptokokken verursachte Mastitis bei Rindern, aus früherer Literatur, bestätigt werden. Jedoch muss auf die Wichtigkeit des verantwortungsvollen Umganges mit Antibiotika hingewiesen werden.

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9. Register of Illustrations

10. List of Tables

11. Appendix

Table 8: Register of isolates

Isolate	Sample date	Milk quarter	Species
1	Dec-15	RV	*Streptococcus dysgalactiae*
2	Dec-15	RH	*Streptococcus dysgalactiae*
3	Nov-15	RH	*Streptococcus uberis*
4	Oct-15	LH	*Streptococcus uberis*
5	Oct-15	LH	*Streptococcus uberis*
6	Oct-15	LH	*Streptococcus uberis*
7	Nov-15	LV	*Streptococcus uberis*
8	Jun-16	LH	*Streptococcus uberis*
9	Feb-16	LH	*Streptococcus parauberis*
10	Sep-16	RV	*Streptococcus uberis*
11	Jan-16	RH	*Streptococcus uberis*
12	Jan-16	LV	*Streptococcus uberis*
13	Feb-16	RH	*Streptococcus uberis*
14	Nov-15	RH	*Streptococcus uberis*
15	Sep-16	RH	*Streptococcus uberis*
16	Jun-16	LH	*Streptococcus uberis*
17	Aug-16	RH	*Streptococcus uberis*
18	Nov-15	LV	*Streptococcus uberis*
19	Oct-15	RH	*Streptococcus dysgalactiae*
20	Nov-15	LH	*Streptococcus uberis*
21	May-16	LV	*Streptococcus dysgalactiae*
22	May-16	LH	*Streptococcus pluranimalium*
23	May-16	LV	*Streptococcus uberis*
24	Nov-15	RH	*Streptococcus uberis*
25	Sep-16	RH	*Streptococcus uberis*
26	Sep-16	RV	*Streptococcus uberis*
27	Aug-16	RH	*Streptococcus dysgalactiae*
28	Apr-16	RH	*Streptococcus uberis*
29	Feb-16	RH	*Streptococcus dysgalactiae*
30	Oct-15	RH	*Streptococcus parauberis*
31	Feb-16	RV	*Streptococcus dysgalactiae*
32	Aug-16	RH	*Streptococcus uberis*
33	Mar-16	RV	*Streptococcus uberis*
34	Dec-15	RH	*Streptococcus uberis*

35	Nov-15	LV	*Streptococcus uberis*
36	Dec-15	LH	*Streptococcus uberis*
37	Jun-16	RV	*Streptococcus uberis*
38	Aug-16	LV	*Streptococcus uberis*
39	Nov-15	RV	*Streptococcus uberis*
40	Jul-16	RH	*Streptococcus uberis*
41	Oct-15	RH	*Streptococcus uberis*
42	Nov-15	LH	*Streptococcus uberis*
43	Mar-16	RH	*Streptococcus dysgalactiae*
44	Apr-16	LV	*Streptococcus dysgalactiae*
45	Jul-16	RV	*Streptococcus uberis*
46	Dec-15	RH	*Streptococcus uberis*
47	Dec-15	RH	*Streptococcus dysgalactiae*
48	Dec-15	LV	*Streptococcus dysgalactiae*
49	Nov-15	RH	*Streptococcus uberis*
50	Jan-16	LH	*Streptococcus uberis*
51	Nov-15	LH	*Streptococcus salivarius*
52	Jan-16	LH	*Streptococcus dysgalactiae*
53	Oct-15	LH	*Streptococcus uberis*
54	Jul-16	LH	*Streptococcus uberis*
55	May-16	LH	*Streptococcus dysgalactiae*
56	Jan-16	RH	*Streptococcus dysgalactiae*
57	Mar-16	LH	*Streptococcus uberis*
58	Oct-15	LH	*Streptococcus uberis*
59	Feb-16	RV	*Streptococcus dysgalactiae*
60	Jun-16	RH	*Streptococcus dysgalactiae*
61	Apr-16	LH	*Streptococcus uberis*
62	Jun-16	RV	*Streptococcus uberis*
63	Sep-16	LH	*Streptococcus uberis*
64	Dec-15	LV	*Streptococcus uberis*
65	Sep-16	RH	*Streptococcus uberis*
66	Feb-16	LH	*Streptococcus macedonicus*
67	Jan-16	LH	*Streptococcus dysgalactiae*
68	Nov-15	LV	*Streptococcus uberis*
69	Oct-15	LH	*Streptococcus uberis*
70	Oct-15	LH	*Streptococcus uberis*
71	Apr-16	RV	*Streptococcus uberis*
72	May-16	RH	*Streptococcus uberis*
73	Jun-16	LV	*Streptococcus uberis*

74	Jan-16	LH	*Streptococcus uberis*
75	Apr-16	LH	*Streptococcus dysgalactiae*
76	Oct-15	LV	*Streptococcus uberis*
77	Apr-16	LV	*Streptococcus uberis*
78	Aug-16	RH	*Streptococcus dysgalactiae*
79	Jan-16	RV	*Streptococcus uberis*
80	Sep-16	LH	*Streptococcus dysgalactiae*
81	Aug-16	RV	*Streptococcus dysgalactiae*
82	Sep-16	RV	*Streptococcus uberis*
83	May-16	RV	*Streptococcus uberis*
84	Sep-16	RV	*Streptococcus uberis*
85	Mar-16	LH	*Streptococcus uberis*
86	Nov-15	LH	*Streptococcus dysgalactiae*
87	Oct-15	LH	*Streptococcus uberis*
88	Oct-15	RH	*Streptococcus uberis*
89	Oct-15	LV	*Streptococcus dysgalactiae*
90	Aug-16	LV	*Streptococcus uberis*
91	Jun-16	RV	*Streptococcus uberis*
92	Oct-15	LV	*Streptococcus uberis*
93	Aug-16	LH	*Streptococcus dysgalactiae*
94	Oct-15	RH	*Streptococcus uberis*
95	Feb-16	RH	*Streptococcus pasteurianus*
96	Oct-15	LH	*Streptococcus uberis*
97	Oct-15	RH	*Streptococcus uberis*
98	Sep-16	LV	*Streptococcus uberis*
99	Jul-16	RV	*Streptococcus uberis*
100	Jan-16	RV	*Streptococcus dysgalactiae*
101	Mar-16	LH	*Streptococcus uberis*
102	Dec-15	LH	*Streptococcus uberis*
103	Oct-15	RH	*Streptococcus uberis*
104	Aug-16	RH	*Streptococcus uberis*
105	May-16	RH	*Streptococcus uberis*
106	Apr-16	LV	*Streptococcus dysgalactiae*
107	Jul-16	LV	*Streptococcus uberis*
108	Nov-15	RH	*Streptococcus uberis*
109	Oct-15	LH	*Streptococcus uberis*
110	Dec-15	RH	*Streptococcus dysgalactiae*
111	Nov-15	LH	*Streptococcus uberis*
112	Nov-15	RV	*Streptococcus uberis*

113	Oct-15	RH	*Streptococcus uberis*
114	Nov-15	RV	*Streptococcus uberis*
115	Apr-16	RV	*Streptococcus dysgalactiae*
116	Aug-16	RH	*Streptococcus uberis*
117	Oct-15	RV	*Streptococcus uberis*
118	Oct-15	RH	*Streptococcus dysgalactiae*
119	Apr-16	RH	*Streptococcus dysgalactiae*
120	Nov-15	LH	*Streptococcus uberis*
121	Nov-15	RV	*Streptococcus uberis*
122	Jul-16	RV	*Streptococcus equinus*
123	Mar-16	RV	*Streptococcus dysgalactiae*
124	Apr-16	RV	*Streptococcus uberis*
125	Sep-16	RH	*Streptococcus uberis*
126	Jun-16	LH	*Streptococcus uberis*
127	Oct-15	LH	*Streptococcus uberis*
128	Oct-15	RH	*Streptococcus uberis*
129	Dec-15	LH	*Streptococcus uberis*
130	Jun-16	RH	*Streptococcus uberis*
131	Apr-16	RH	*Streptococcus uberis*
132	Aug-16	LH	*Streptococcus uberis*
133	Sep-16	LH	*Streptococcus uberis*
134	Feb-16	RV	*Streptococcus uberis*
135	Feb-16	RH	*Streptococcus pasteurianus*
136	Sep-16	LH	*Streptococcus dysgalactiae*
137	Jan-16	RH	*Streptococcus uberis*
138	Jul-16	LV	*Streptococcus uberis*
139	May-16	RH	*Streptococcus uberis*
140	Nov-15	LH	*Streptococcus uberis*
141	Nov-15	RV	*Streptococcus dysgalactiae*
142	Feb-16	RV	*Streptococcus dysgalactiae*
143	Mar-16	RV	*Streptococcus uberis*
144	Nov-15	RH	*Streptococcus uberis*
145	Jul-16	RV	*Streptococcus uberis*
146	Jan-16	LV	*Streptococcus uberis*
147	Aug-16	LH	*Streptococcus uberis*
148	Dec-15	LH	*Streptococcus uberis*
149	Aug-16	LV	*Streptococcus uberis*
150	Aug-16	RH	*Streptococcus uberis*
151	Aug-16	LH	*Streptococcus uberis*

152	Nov-15	RH	*Streptococcus macedonicus*
153	Apr-16	RH	*Streptococcus uberis*
154	Nov-15	RH	*Streptococcus dysgalactiae*
155	May-16	LH	*Streptococcus uberis*
156	Nov-15	RV	*Streptococcus uberis*
157	Jun-16	LV	*Streptococcus lutetiensis*
158	Jun-16	LH	*Streptococcus dysgalactiae*
159	Feb-16	RV	*Streptococcus dysgalactiae*
160	Apr-16	RH	*Streptococcus dysgalactiae*
161	Dec-15	RV	*Streptococcus uberis*
162	Dec-15	LH	*Streptococcus dysgalactiae*
163	Nov-15	RH	*Streptococcus pluranimalium*
164	May-16	LV	*Streptococcus uberis*
165	Aug-16	RV	*Streptococcus uberis*
166	Jan-16	LV	*Streptococcus uberis*
167	Oct-15	LH	*Streptococcus uberis*
168	Oct-15	RH	*Streptococcus uberis*
169	Jan-16	LH	*Streptococcus dysgalactiae*
170	Jul-16	RH	*Streptococcus dysgalactiae*
171	Sep-16	LV	*Streptococcus uberis*
172	Dec-15	LH	*Streptococcus uberis*
173	Dec-15	LH	*Streptococcus uberis*
174	Jan-16	RH	*Streptococcus dysgalactiae*
175	Oct-15	LH	*Streptococcus dysgalactiae*
176	Jan-16	RH	*Streptococcus pluranimalium*
177	Feb-16	RV	*Streptococcus uberis*
178	Apr-16	LH	*Streptococcus dysgalactiae*
179	Oct-15	LV	*Streptococcus dysgalactiae*
180	Jan-16	RH	*Streptococcus dysgalactiae*
181	Oct-15	RV	*Streptococcus dysgalactiae*
182	Oct-15	LV	*Streptococcus dysgalactiae*
183	Jun-16	RV	*Streptococcus dysgalactiae*
184	Jan-16	RV	*Streptococcus uberis*
185	Nov-15	RH	*Streptococcus uberis*
186	Sep-16	RV	*Streptococcus uberis*
187	Sep-16	RH	*Streptococcus uberis*
188	May-16	LH	*Streptococcus dysgalactiae*
189	May-16	LH	*Streptococcus dysgalactiae*
190	Aug-16	RH	*Streptococcus uberis*

191	Feb-16	LH	*Streptococcus uberis*
192	Apr-16	RV	*Streptococcus uberis*
193	Nov-15	RV	*Streptococcus uberis*
194	Dec-15	LH	*Streptococcus uberis*
195	Feb-16	LV	*Streptococcus dysgalactiae*
196	Jul-16	RV	*Streptococcus dysgalactiae*
197	Nov-15	LH	*Streptococcus uberis*
198	Apr-16	RV	*Streptococcus dysgalactiae*
199	Feb-16	RH	*Streptococcus uberis*
200	Feb-16	LV	*Streptococcus dysgalactiae*
201	Oct-15	LV	*Streptococcus dysgalactiae*
202	Jan-16	RV	*Streptococcus uberis*
203	Aug-16	LH	*Streptococcus dysgalactiae*
204	Sep-16	LH	*Streptococcus uberis*
205	Nov-15	RH	*Streptococcus uberis*
206	Aug-16	RH	*Streptococcus uberis*
207	Aug-16	LH	*Streptococcus dysgalactiae*
208	Oct-15	RH	*Streptococcus dysgalactiae*
209	Oct-15	LH	*Streptococcus uberis*
210	Sep-16	RV	*Streptococcus uberis*
211	Oct-15	RH	*Streptococcus uberis*
212	Oct-15	RV	*Streptococcus uberis*
213	Jan-16	LV	*Streptococcus dysgalactiae*
214	Feb-16	RH	*Streptococcus dysgalactiae*
215	May-16	RH	*Streptococcus dysgalactiae*
216	Jun-16	RH	*Streptococcus uberis*
217	Oct-15	RH	*Streptococcus uberis*
218	Feb-16	RV	*Streptococcus uberis*
219	May-16	LH	*Streptococcus uberis*
220	Feb-16	RH	*Streptococcus dysgalactiae*
221	Apr-16	LV	*Streptococcus dysgalactiae*
222	Feb-16	LH	*Streptococcus dysgalactiae*
223	Oct-15	LH	*Streptococcus parauberis*
224	Jan-16	LV	*Streptococcus uberis*
225	Jan-16	RH	*Streptococcus pluranimalium*
226	Sep-16	RV	*Streptococcus uberis*
227	Jan-16	RH	*Streptococcus uberis*
228	Nov-15	RV	*Streptococcus uberis*
229	Sep-16	LV	*Streptococcus dysgalactiae*

230	Aug-16	RH	*Streptococcus dysgalactiae*
231	Sep-16	RV	*Streptococcus dysgalactiae*
232	Jan-16	RH	*Streptococcus uberis*
233	Oct-15	RH	*Streptococcus uberis*
234	Apr-16	LV	*Streptococcus uberis*
235	Oct-15	LH	*Streptococcus uberis*
236	Aug-16	RV	*Streptococcus uberis*
237	Aug-16	LH	*Streptococcus uberis*
238	May-16	RH	*Streptococcus dysgalactiae*
239	Nov-15	RV	*Streptococcus equi*
240	Jan-16	RV	*Streptococcus dysgalactiae*
241	Sep-16	RH	*Streptococcus uberis*