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Veterinary Health Service in Upper Austria: Dairy farms and bovine quarter milk sample analysis from 2011 to 2014 – an overview

Oberösterreichischer Tiergesundheitsdienst: Milchviehbetriebe und Viertelgemelksprobenuntersuchung zwischen 2011 und 2014 – ein Überblick

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Summary

The Veterinary Health Service („Tiergesundheitsdienst", TGD), an organisation of veterinarians and farmers, has been established for all federal states of Austria but Vienna. Here we present data concerning Upper Austrian dairy farms and results of bacteriological analysis of bovine quarter milk samples from the Upper Austrian TGD laboratory between 2011 and 2014, especially relating to prevalence and antibiotic susceptibilities of mastitis pathogens.
Microbial isolates from quarter milk samples of Austrian cows were cultivated and identified using classical and advanced methods and tested for antibiotic susceptibility by the microdilution method.
Generally, there has been a decrease in the number of milk suppliers and TGD farms in Upper Austria. Almost 10500 milk samples were analysed at the Upper Austrian TGD laboratory each year. Most commonly isolated udder pathogens in quarter milk samples were *Staphylococcus aureus* (26%), *Streptococcus uberis* (22%) and non-aureus staphylococci (15%). For many antibiotic agents we found high antimicrobial susceptibility of tested isolates. However, also an increasing prevalence of resistant strains against the beta-lactam antibiotics and the macrolide tylosin was detected. The mean proportion of multi-resistant isolates in staphylococci and streptococci isolates was below 5%.
This longitudinal study acknowledges changes in the structure of Austrian dairies, the occurrence of mastitis pathogens and their antibiotic susceptibilities. Such adaptations could suggest the need for new types of farm management, together with preventive and treatment strategies.

Keywords: TGD, Mastitis, pathogen, resistance, udder

Zusammenfassung

Die Organisation des Tiergesundheitsdienstes (TGD) inkludiert TierärztInnen und LandwirtInnen sowie eigene diagnostische Laboratorien und ist mittlerweile österreichweit in jedem Bundesland außer Wien eingerichtet. In dieser Studie werden, neben allgemeinen Daten zu den oberösterreichischen milchliefernden Betrieben, Ergebnisse der bakteriologischen Untersuchung von bovinen Viertelgemelksproben des oberösterreichischen TGD Labors zwischen 2011 und 2014 präsentiert.
Die bakteriologische Milchuntersuchung inkludierte die Identifizierung der Mikroorganismen anhand Kultivierung, Analyse mit klassischen und spezifischen mikrobiologischen Testsystemen sowie die Bestimmung der Antibiotika-Empfindlichkeit mittels Mikrodilutionsmethode.
Die Anzahl der milchliefernden Betriebe in Oberösterreich nahm um 12 % von 2011–2014 ab. Circa 10500 Milchproben wurden pro Jahr im TGD Labor analysiert. Die am häufigsten detektierten Euterpathogene waren *Staphylococcus aureus* (26 %), *Streptococcus uberis* (22 %) und nicht-aureus Staphylokokken (15 %). Für sehr viele antibiotische Wirkstoffe wurde eine hohe antimikrobielle Wirkung gegenüber den vorherrschenden Pathogenen nachgewiesen, jedoch wurde auch eine zunehmende

Anzahl resistenter Isolate gegen bestimmte Wirkstoffe wie Beta-Lactam Antibiotika und dem Makrolid Tylosin gefunden. Der durchschnittliche Anteil multiresistenter Staphylokokken und Streptokokken war kleiner als 5 %.
Diese Studie zeigt Änderungen in der Struktur der Milchwirtschaft und der detektierten Euterpathogene bezüglich Spezies und Antibiotikaresistenzen innerhalb von vier Jahren. Obwohl dies keine allgemeine Prävalenzstudie ist und Proben nur eines Bundeslandes umfasst, weisen diese Daten, vor allem die detektierten Euterpathogene und deren ansteigende Antibiotikaresistenz, darauf hin, dass neue Management- und Präventionsstrategien auf Betriebs- als auch auf veterinärmedizinischer (therapeutischer) Ebene verlangt werden.

Schlüsselwörter: TGD, Mastitis, Pathogen, Resistenz, Euter

Introduction

With the exception of Vienna, Austria has introduced veterinary health services ("Tiergesundheitsdienste", TGDs) for each federal state (Österreichischer Tiergesundheitsdienst, 2016). The TGD is a permanently established organisation of veterinarians and animal farmers that aims to reduce the use of veterinary drugs and prevent deficiencies in livestock farming due to inadequate husbandry. These veterinary health services thereby aspire to maximize animal health, food quality and safety and overall quality of products for consumers. TGD membership necessitates adherence to certain legalizations and contractual regulations (Tiergesundheitsdienst-Verordnung 2009). Compared to other animal husbandries in the country, cattle farmers are most represented in the TGD organisation. In 2016, 42.1% of all Austrian cattle farmers were TGD members (Tiergesundheitsdienst 2016). The established TGD laboratories provide a wide variety of investigations for veterinary samples, including the bacteriological analysis of bovine quarter milk samples (Oberösterreichischer Tiergesundheitsdienst 2016).

Bovine mastitis, an inflammation of the udder, occurs worldwide, challenging the dairy industry with significant economic losses. These include reduced milk yield, increased morbidity and mortality of dairy cows and their associated veterinary costs. Further, bovine mastitis has a high zoonotic potential due to the shedding of bacteria and their toxins in milk (Abebe et al. 2016). Although over 100 species of mastitis pathogens are now recognized, streptococci, coliforms and staphylococci are most commonly detected (Szweda et al. 2014).

It has been shown that the antimicrobial consumption in food-producing animals and also in humans results in bacterial resistance (European Centre for Disease Prevention and Control et al. 2017). Resultantly, specific antibiotic resistance, multi-resistance and the presumed transfer of the genetic elements encoding resistance between organisms are significant causes for concern (Bennedsgaard et al. 2006, Lord Soulsby of Swaffham Prior 2008). Most alarmingly, bacterial strains of various species from diseased animals, including methicillin-resistant *Staphylococcus* (*S.*) *aureus* (MRSA) and extend-spectrum beta-lactamase (ESBL)-producing *Escherichia* (*E.*) *coli*, are resistant to antibiotics commonly used in human medicine (Weiner et al. 2015). Therefore, in order to safeguard human and animal health through preventative strategies, it is important to conduct regular bacteriological analyses in specialised veterinary laboratories and monitor resistance levels (Makovec and Ruegg 2003). Investigations on pathogens isolated from animals in the course of monitoring programs are thereby important steps for the early detection of spreading resistance (Wallmann et al. 2003).

We present here data from dairy farms in Upper Austria and results of bovine quarter milk sample analysis by the Upper Austrian TGD laboratory from 2011 to 2014. This longitudinal study included the occurrence and antibiotic resistance levels of the six most common udder pathogens identified during this time.

Materials and methods

Data

Data from Upper Austrian dairy farms was obtained by Agrarmarkt Austria Marketing (AMA) and the Veterinary Information System (VIS). Quarter milk sample analysis data was provided by the Upper Austrian veterinary health service laboratory. Data on the six most common pathogens were included in this study. Excluded was data on antibiotic susceptibility testing of *Enterococcus* (*E.*) *faecalis* to the lincosamides lincomycin and pirlimycin due to intrinsic resistance of this species to these agents (Pyörälä et al. 2014).

Samples

Bovine quarter milk samples were collected aseptically by veterinarians or milk suppliers and sent for analysis to the Upper Austrian TGD laboratory (Ried im Innkreis, Upper Austria) in special milk sample tubes. Usually all four udder quarters of cows were sampled, making four separate milk samples per cow (quarter milk samples). Submissions per farm sometimes also included samples of more than one animal. Prior to microbiological investigations at the TGD laboratory, broken tubes were discarded, samples were visually inspected for changed visual characteristics and then homogenized. All deviations from physiological appearance were recorded.

Microbiological cultivation

The bacteriological analysis of quarter milk samples including the classification and differentiation of udder pathogens and the determination of antimicrobial susceptibility was based on the announcement of the modul "Eutergesundheit" of the Bundesministerium für Gesundheit und Frauen (2005). Therefore, samples' background information on animal and udder health such as clinical factors and somatic cell count (data not shown) were included for the interpretation of the results of bacteriological analysis and the classification of udder pathogens.

Columbia agar plates supplemented with sheep blood (COS; Columbia agar with 5% sheep blood Biomerieux, Marcy l'Etoile, France) were inoculated with quarter milk samples using sterile loops and incubated aerobically at 37 °C for 48 hours. The agar plates were briefly inspected after 24 hours for bacterial growth. If clearly identifiable contamination was detected (overgrowth of highly heterogeneous cultures; not evaluable), samples were excluded as "contaminated". If there was no microbial growth after 24 hours, additional cytological investigation was performed. This involved centrifugation of the milk sample (3500 rpm, 5 min, room temperature), discarding of the supernatant, visual assessment of the sediment, smearing the sediment onto microscope slides and drying by air and heat fixation, followed by Gram-staining and microscopic examination under oil immersion. Neutrophilic granulocytes were counted. If more than ten neutrophils were present, the milk sediment was enriched. For this, a cotton swab of the milk sediment was used to inoculate trypcase-soja-bouillon (Bouillon Trypcase Soja, Biomerieux) and tubes incubated for 24 hours at 37 °C. Subsequently, sediment enrichment was visually inspected, COS agar plates inoculated and incubated at 37 °C for 24 hours. If no microbial growth was observed after the total incubation period of 48 hours, no further analysis was performed and samples were recorded as "no microbiological growth". If two or more colonies were present that had different and difficult to distinguish morphologies, subcultures were prepared and included in the microbiological identification analysis. Clearly distinguishable colonies were selected for further investigations.

Microbiological identification

Microbiological identification involved the use of classical methods (evaluation of colony morphology, i.e. color, size and shape, the presence of hemolysis, catalase test, Gram-staining, responses to KOH- (potassium hydroxide), indole- and oxidase tests) as well as advanced commercially available testing systems. These included the clumping factor test (*Staphylococcus* species: SLIDEX® Staph Kit, Biomerieux), Lancefield grouping (*Streptococcus* species: SLIDEX® StreptoPlus, Biomerieux), and cultivation on selective agar plates (*E. coli*: BBL™ CHROMagar™ Orientation, Becton Dickinson, Franklin Lakes, New Jersey). For *Enterobacteriaceae* other than *E. coli* (*Citrobacter*, *Klebsiella*, and *Serratia* spp.), additionally, an API® (API® RAPID 20E, Biomerieux) identification test was performed. *Trueperella* and *Bacillus* species were identified using the BD BBL™ Crystal Gram-positive ID kit (BD BBL™ Crystal Gram-positive ID kit, Becton Dickinson). *Staphylococcus* species, negative or unclear in the clumping factor test, were further included in the BD BBL™ Crystal™ Gram-positive ID test or API® Staph test (API® STAPH, Biomerieux). BD BBL™ Crystal™ Gram-positive ID (BD BBL™ Crystal™ Gram-positive ID kit, Becton Dickinson) or API® RAPID ID 32 STREP tests (API® RAPID ID 32 STREP, Biomerieux) were performed for non-groupable or unclassified *Streptococcus* species. Furthermore, a CAMP-test evaluating hemolysis was performed for *Streptococcus* (*S.*) *agalactiae* isolates.

Antibiotic susceptibility

Antibiotic susceptibility was determined by the microdilution method using the Micronaut-susceptibility testing system (Micronaut-S, MERLIN Diagnostika GmbH, Bornheim-Hersel, Germany). The procedure recommended by the manufacturer involved rehydration of antimicrobials in 96-well-microtiter plates by the addition of standardised bacterial suspensions of specific bacterial media: Mueller-Hinton II Bouillon (Mueller-Hinton II Bouillon, MERLIN Diagnostika GmbH) and H-Medium (H-Medium for fastidious organisms, MERLIN Diagnostika GmbH) for fastidious organisms. Media were incubated at 35–37 °C for 18–24 hours, after which photometric measurement of the optical density (as a measure of bacterial growth) was performed with the Skan device and results evaluated with MICRONAUT software (integrated standardised annually valid CLSI (Clinical and Laboratory Standards Institute®) breakpoints). Two different Micronaut plates were used for antibiotics for Gram-positive and Gram-negative organisms. Antimicrobials for Gram-positive bacteria were amoxicillin-clavulanic acid, ampicillin, cefalexine, cefazolin, cefoperazone, cefquinome, cloxacillin, enrofloxacin, lincomycin, penicillin G, pirlimycin, rifaximin and tylosin. Antimicrobials for Gram-negative bacteria were amoxicillin-clavulanic acid, ampicillin, cefalexine, cefazolin, cefoperazone, cefquinome, danofloxacin, enrofloxacin, kanamycin, tetracycline and trimethoprim/sulfamethoxazole. Bacterial isolates were classified as either "susceptible" (S) or "resistant" (R). The categorisation "intermediate" (I) was not used due to its lack of practical relevance in respect of animal treatment. Therefore, all isolates showing intermediate antibiotic susceptibility were classified as "resistant".

Statistical analysis

Statistical analysis was performed using R version 3.1.3 (R Development Core Team 2008). All raw data (pertaining to farms, veterinarians and the TGD laboratory) was provided by the Upper Austrian TGD. To calculate the prevalence of resistant and multi-resistant bacteria, the antibiotic agents were grouped into antimicrobial classes, according to the scheme of the Clinical and Laboratory Standards Institute® (CLSI 2012). Multi-resistance was defined as resistance against three or more antibiotic classes.

Results

Upper Austrian farms and veterinarians

The total number of Upper Austrian milk suppliers, as well as the farms registered and included in the TGD program (TGD farms), decreased between 2011 and 2014. This change equates with a decrease of 1401 (12%) milk suppliers and 547 (8%) TGD farms over the four years: Table 1 shows that in contrast to the number of farms, the median number of dairy cows per milk supplier and also per TGD farm increased between 2011 and 2014. Further, there was also a decrease (7%) in the number of supporting TGD veterinarians during this period.

Samples

Each year between 2011 and 2014, around 10500 quarter milk samples were submitted to the Upper Austrian TGD laboratory. The highest number of submissions was made in 2012, whereas the highest number of samples was analyzed in 2013 (Table 1). Since submissions from farms also included quarter milk samples from more than one animal, the number of submissions did not equate with the number of total samples.

Microbiological analysis and distribution of pathogens

Of submitted samples, 55.7% were classified as positive (microbial growth) and 44.3% as negative (no microbial growth after the prescribed incubation period; Table 2). The percentage of positive samples (+ 7.2%) increased over the four years. The distribution of the six most frequently detected udder pathogens is shown in Table 3. *Staphylococcus aureus* was the most frequently detected pathogen in investigated samples over all four years, followed by *Streptococcus* (*S.*) *uberis*, non-aureus staphylococci (NAS), *Streptococcus* (*S.*) *dysgalactiae*, *E. coli* and *E. faecalis*. The highest occurrence of *S. aureus* was in 2013, whereas NAS, *S. dysgalactiae* and *E. coli* had their highest occurrence in 2014. In contrast to *S. uberis*, for which fewer isolates were detected in 2013 and 2014, the number of NAS isolates found during these years was higher than in 2011 and 2012.

Proportion of pathogen-positive quarter samples per cow

For submissions that included all four separate udder quarter samples of one cow, we also evaluated the proportion of pathogen-positive quarter milk samples per animal (Figure 1). Infections of single udder quarters were dominated by *E. coli*: in over 90% of cases only one quarter milk sample was *E.coli*-positive. Similarly, infections with *S. dysgalactiae* and *S. uberis* were mainly confined to only one quarter milk sample per cow. *S. aureus* was found more frequently in two or more quarter milk samples per cow, which was the case in over 30% of *S. aureus*-positive udders. Likewise, non-aureus staphylococci, followed by *E. faecalis*, were frequently detected in two or more udder quarters.

Antibiotic resistance

Staphylococcus aureus

Over 15% of the *S. aureus* isolates tested were resistant to the beta-lactam agents penicillin G and ampicillin (Table 4). A lower number of resistant isolates was found for beta-lactamase-stable cloxacillin and amoxicillin-clavulanic acid (< 4% resistant isolates between 2011 and 2014). Further, a great proportion of *S. aureus* isolates were susceptible for all tested cephalosporins, lincosamide and fluoroquinolones (< 4% resistant isolates). Particularly striking was that the number of tylosin-resistant *S. aureus* increased in 2013 and 2014 compared to 2011 and 2012.

Non-aureus staphylococci

As with *S. aureus*, NAS isolates had highest resistance to penicillin G and ampicillin over the four years (mean 23.8 ± 4.0% and 18.5 ± 3.6% resistant isolates). The average number of resistant isolates to tylosin was 20.3 ± 4.6%, but only less than 2.5% of isolates were resistant to beta-lactamase-stable agents (Table 5).

Streptococci (Streptococcus uberis, Streptococcus dysgalactiae)

Less than 11% of streptococcal isolates tested were resistant to beta-lactam antibiotics. For most of these antimicrobials, resistant isolates increased between 2011 and 2014 (Table 6, Table 7). For *S. dysgalactiae*, the

TABLE 1: *Numbers of Upper Austrian milk suppliers, TGD-farms and veterinarians, dairy cattle, submissions and samples.*

	2011	2012	2013	2014	Mean ± SD[a]
Milk suppliers (Upper Austria), total	11614	11012	10739	10213	10895 ± 583
TGD- farms (Upper Austria), total	6780	6616	6359	6233	6497 ± 247
Sampled TGD-farms, total	1816	1979	1956	1935	1922 ± 73
TGD Veterinarians, total	203	201	183	188	194 ± 10
Herd size in milk supplier farms, median	30	31	31	32	
Herd size in TGD farms, median	41	42	43	44	
Submissions, total	4732	5360	5284	5228	5151 ± 285
Samples, total	9521	10907	11062	10462	10488 ± 693

TGD: organisation of the Veterinary Health Service („Tiergesundheitsdienst")
a Mean, standard deviation (± SD)

TABLE 2: *Numbers (N) of submitted samples showing microbial growth (positive samples) and no microbial growth (negative samples).*

Year	Number of samples	Positive N (%)	Negative N (%)
2011	8689	4675 (53.8)	4014 (46.2)
2012	9810	5364 (54.7)	4446 (45.3)
2013	9758	5193 (53.2)	4565 (46.8)
2014	9361	5714 (61.0)	3647 (39.0)
Mean	**9405**	**5236 (55.7)**	**4168 (44.3)**

TABLE 3: *Distribution (%) of the six most common udder pathogens between 2011 and 2014 in positive samples.*

	2011	2012	2013	2014	Mean ± SD[a]
Staphylococcus aureus	25.6	25.4	29.1	23.6	25.9 ± 2.3
Streptococcus uberis	23.2	22.9	19.5	21.2	21.7 ± 1.7
NAS	14.3	13.4	16.0	17.5	15.3 ± 1.8
Streptococcus dysgalactiae	12.3	13.2	11.7	13.7	12.7 ± 0.9
Escherichia coli	7.3	7.0	6.0	7.5	7.0 ± 0.7
Enterococcus faecalis	3.1	4.4	4.2	3.6	3.8 ± 0.6

a Mean, standard deviation (± SD)

TABLE 4: *Occurrence (%) of antimicrobial resistant Staphylococcus aureus isolates.*

Antimicrobial class[a]	Antimicrobial agent	2011	2012	2013	2014	Mean ± SD[b]
Penicillins	Penicillin G	20.9	20.7	17.1	20.5	**19.8 ± 1.8**
	Ampicillin	19.0	19.2	15.3	16.8	**17.6 ± 1.9**
	Cloxacillin	2.4	2.2	2.2	3.9	**2.7 ± 0.8**
β-Lactam/β-Lactamase Inhibitor Combinations	Amoxicillin-clavulanic acid	0.1	0.1	0.5	0.1	**0.2 ± 0.2**
Cephems (including cephalosporin)	Cefalexine	1.0	0.8	1.6	2.9	**1.5 ± 1.0**
	Cefazolin	0.6	0.1	0.5	1.2	**0.6 ± 0.5**
	Cefoperazone	1.1	0.6	2.2	2.9	**1.7 ± 1.1**
	Cefquinome	0.5	0.1	0.8	2.2	**0.9 ± 1.0**
Macrolides	Tylosin	10.5	7.1	16.8	17.8	**13.1 ± 5.1**
Quinolones	Enrofloxacin	0.2	0.4	0.4	0.9	**0.5 ± 0.3**
Lincosamides	Lincomycin	1.7	0.9	1.9	3.3	**1.9 ± 1.0**
	Pirlimycin	1.3	1.3	2.0	2.7	**1.8 ± 0.6**
Ansamycins	Rifaximin	1.2	1.5	1.1	1.5	**1.3 ± 0.2**

a classification of antimicrobials to antimicrobial classes, according to Clinical and Laboratory Standards Institute (MA07-A9, 2012)
b Mean, standard deviation (± SD)

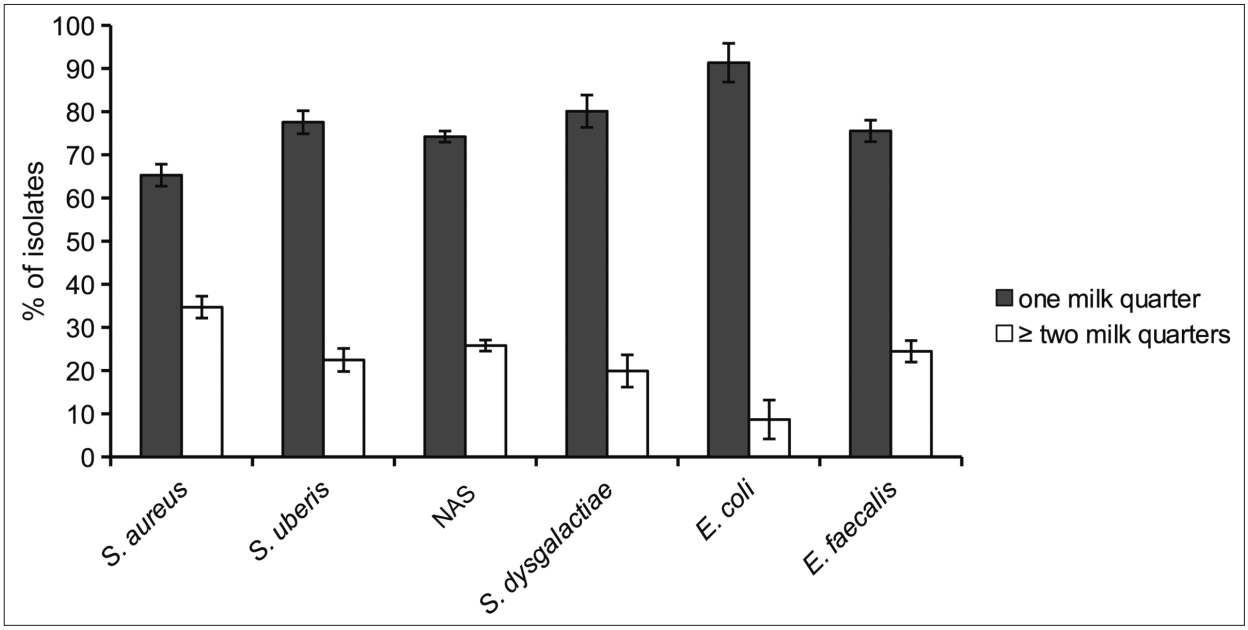

FIGURE 1: *Frequency of the six most common udder pathogens (%) detected in only one udder quarter sample or more than two udder quarters per cow.*

greatest increase in resistant isolates was found for penicillin G (+ 8%) and cloxacillin (+ 9%, Table 7). Tylosin susceptibility was found to be quite variable for the two streptococci species. *S. uberis* isolates were highly resistant (mean 49.2 ± 8.9% resistant isolates) compared with *S. dysgalactiae* (mean 9.7 ± 4.1% resistant isolates).

Escherichia coli

We detected a strong increase in the number of *E. coli* isolates resistant to ampicillin, up from 17 to 61%, and amoxicillin-clavulanic acid, up from 3 to 59%, between 2011 and 2014 (Table 8). Further, the highest number of detected resistant isolates to all cephalosporins was found in 2014. Less than 8% of isolates were resistant to the fourth-generation cephalosporin cefquinome. Resistance levels were 23% for tetracycline and 18% for kanamycin in 2014. Enrofloxacin resistance was detected in < 4% of isolates from 2011 to 2014 and the change over the four year period was quite low (SD: ± 0.70).

Enterococcus faecalis

There was a high proportion of *E. faecalis* isolates resistant to almost all tested antibiotics (> 50% resistant isolates) with the exception of amoxicillin-clavulanic acid, enrofloxacin and cefquinome, over all four years (Table 9). Susceptibility to the combination of amoxicillin-clavulanic acid was especially high and few resistant isolates were detected (< 2%). There was a generally high susceptibility to enrofloxacin among *E. faecalis* isolates compared to other antibiotics, although number of enrofloxacin-resistant isolates increased between 2011 and 2014 from 9.2 to 25.5%.

TABLE 5: *Occurrence (%) of antimicrobial resistant non-aureus staphylococci (NAS) isolates.*

Antimicrobial class[a]	Antimicrobial agent	2011	2012	2013	2014	Mean ± SD[b]
Penicillins	Penicillin G	24.2	18.5	28.3	24.1	**23.8 ± 4.0**
	Ampicillin	19.9	15.3	23.0	16.0	**18.5 ± 3.6**
	Cloxacillin	1.4	1.9	3.6	3.0	**2.4 ± 1.0**
β-Lactam/β-Lactamase Inhibitor Combinations	Amoxicillin-clavulanic acid	0.1	0.0	1.2	1.0	**0.6 ± 0.6**
Cephems (including cephalosporins)	Cefalexine	0.3	0.3	0.8	0.8	**0.5 ± 0.3**
	Cefazolin	0.1	0.3	0.5	0.4	**0.3 ± 0.2**
	Cefoperazone	0.8	0.4	1.6	0.8	**0.9 ± 0.5**
	Cefquinome	0.3	0.3	0.9	1.3	**0.7 ± 0.5**
Macrolides	Tylosin	18.0	15.2	25.3	22.8	**20.3 ± 4.6**
Quinolones	Enrofloxacin	0.0	0.4	0.6	1.0	**0.5 ± 0.4**
Lincosamides	Lincomycin	2.3	1.6	4.5	5.9	**3.6 ± 2.0**
	Pirlimycin	1.9	1.2	2.6	2.8	**2.1 ± 0.7**
Ansamycins	Rifaximin	1.1	0.6	1.5	1.4	**1.1 ± 0.4**

a classification of antimicrobials to antimicrobial classes, according to Clinical and Laboratory Standards Institute (Table 4–9, 2012)

b Mean, standard deviation (± SD)

Antibiotic multi-resistance

On average, ≤ 4% of staphylococci and streptococci isolates were multi-resistant. The number of multi-resistant isolates was highest in 2014 for all species, with the exception of non-aureus staphylococci, when it was most common in 2013 (Table 10). A moderately high number of multi-resistant *E. coli* isolates (16% isolates) was found between 2011 and 2013. The number of multi-resistant *E. coli* isolates increased by 26% between 2013 and 2014. The multi-resistant bacteria were particularly represented by *E. faecalis* isolates, which exhibited high multi-resistance over all four years, and included ≥ 92% of isolates resistant to three or more antibiotic classes.

Discussion

In this 4-year longitudinal study we present data on the occurrence of pathogens and their antibiotic resistance of bovine quarter

milk samples. Although this study was geographically limited to one federal state it presents important trends regarding antibiotic resistance.

Generally, the number of both milk suppliers and farms decreased over the recording period. This is likely part of a larger trend in the country where the number of dairy farms is decreasing each year, while at the same time the number of cows per farm is increasing. Between 2003 and 2012 there was a reduction of over 20000 dairy farms in Austria (Kirner et al. 2015). However, there was only a slight increase in the herd size per farm, between 1–3 cows.

The number of samples, submissions and microbiological positive samples increased during the recording period. This increase can be attributed to the quality control program of the Agrarmarkt Austria Marketing (AMA) QS Milch between 2011 and 2014, which introduced obligatory examinations of milk samples having more than 400000 cells/ml and strongly positive California mastitis tests (CMT) three times in succession (Agrarmarkt Austria Marketing GesmbH 2011).

The distribution of mastitis pathogens in the bovine quarter samples was clearly dominated by Gram-positive bacteria, with *S. aureus* being the most common species, followed by *S. uberis* and non-aureus staphylococci. *Staphylococcus aureus* is known to be one of the primary agents responsible for bovine mastitis, including clinical as well as subclinical mastitis. Its special abilities to form biofilms and to hide within host phagocytes and epithelial cells of the mammary gland might enable it to evade antibiotics, leading to prolonged therapy or persistence (Bardiau et al. 2014, Yu et al. 2016). The investigated bovine quarter milk samples derived from all possible mastitis forms (clinical and subclinical mastitis) either diagnosed with mastitis or sampled for control purpose. Therefore, the observed frequencies for *S. aureus* are not surprising considering its association with a wide variety of mastitis presentations.

Besides *S. aureus*, non-aureus staphylococci were commonly identified and their prevalence increased over the four year period. Interestingly, the classification of this bacteria group as udder pathogens with pathogenic potential is not consistent between studies. While in some studies these staphylococci are classified as minor mastitis pathogens or commensal bacteria with absent or restricted pathogenic potential, other studies consider them as true mastitis pathogens (Isaac et al 2017, Pyörälä and Taponen 2009). However, in some countries they are already the most common agents associated with bovine mastitis (Bal et al. 2010, Pyörälä and Taponen 2009).

Streptococcus uberis, a major *Streptococcus* species causing udder inflammation worldwide, and also the pathogen most frequently

TABLE 6: *Occurrence (%) of antimicrobial resistant Streptococcus uberis isolates.*

Antimicrobial class[a]	Antimicrobial agent	2011	2012	2013	2014	Mean ± SD[b]
Penicillins	Penicillin G	2.6	3.1	3.3	8.8	**4.5 ± 2.9**
	Ampicillin	1.6	1.6	1.0	1.9	**1.6 ± 0.4**
	Cloxacillin	1.9	2.3	2.0	5.7	**3.0 ± 1.9**
β-Lactam/β-Lactamase-Inhibitor Combinations	Amoxicillin-clavulanic acid	0.1	0.0	0.0	0.4	**0.1 ± 0.2**
Cephems (including cephalosporins)	Cefalexine	0.7	0.9	0.5	2.4	**1.1 ± 0.9**
	Cefazolin	0.4	0.4	0.5	1.2	**0.6 ± 0.4**
	Cefoperazone	0.7	0.7	0.5	1.8	**0.9 ± 0.6**
	Cefquinome	0.2	0.1	0.1	0.7	**0.3 ± 0.3**
Macrolides	Tylosin	44.4	46.2	62.4	43.7	**49.2 ± 8.9**
Quinolones	Enrofloxacin	0.3	0.2	0.5	3.3	**1.1 ± 1.5**
Lincosamides	Lincomycin	6.3	5.7	10.3	13.1	**8.8 ± 3.5**
	Pirlimycin	5.0	5.7	9.0	10.8	**7.6 ± 2.8**
Ansamycins	Rifaximin	4.7	4.6	4.7	8.2	**5.6 ± 1.7**

a classification of antimicrobials to antimicrobial classes, according to Clinical and Laboratory Standards Institute (Table 4–9, 2012)

b Mean, standard deviation (± SD)

TABLE 7: *Occurrence (%) of antimicrobial resistant Streptococcus dysgalactiae isolates.*

Antimicrobial class[a]	Antimicrobial agent	2011	2012	2013	2014	Mean ± SD[b]
Penicillins	Penicillin G	2.7	3.2	4.8	10.5	**5.3 ± 3.6**
	Ampicillin	1.6	2.1	1.0	2.2	**1.7 ± 0.5**
	Cloxacillin	2.7	3.1	3.2	11.7	**5.2 ± 4.4**
β-Lactam/β-Lactamase Inhibitor Combinations	Amoxicillin-clavulanic acid	0.2	0.0	0.0	0.1	**0.1 ± 0.1**
Cephems (including cephalosporins)	Cefalexine	0.8	1.0	0.3	2.7	**1.2 ± 1.1**
	Cefazolin	0.3	0.3	0.2	1.0	**0.4 ± 0.4**
	Cefoperazone	0.2	0.5	0.1	0.9	**0.4 ± 0.3**
	Cefquinome	0.3	0.0	0.3	1.2	**0.5 ± 0.5**
Macrolides	Tylosin	6.5	7.5	9.3	15.5	**9.7 ± 4.1**
Quinolones	Enrofloxacin	1.0	0.1	0.0	4.2	**1.3 ± 2.0**
Lincosamides	Lincomycin	2.1	3.3	3.2	7.6	**4.1 ± 2.4**
	Pirlimycin	1.3	1.7	3.2	6.2	**3.1 ± 2.2**
Ansamycins	Rifaximin	4.0	2.2	3.8	9.2	**4.8 ± 3.1**

a classification of antimicrobials to antimicrobial classes, according to Clinical and Laboratory Standards Institute (Table 4–9, 2012)

b Mean, standard deviation (± SD)

TABLE 8: *Occurrence (%) of antimicrobial resistant Escherichia coli isolates.*

Antimicrobial class[a]	Antimicrobial agent	2011	2012	2013	2014	Mean ± SD[b]
Penicillins	Ampicillin	17.1	13.1	19.0	61.0	**27.6 ± 22.4**
β-Lactam/β-Lactamase Inhibitor Combinations	Amoxicillin-clavulanic acid	3.3	5.1	13.5	58.7	**20.1 ± 26.1**
Cephems (including cephalosporins)	Cefalexine	7.9	7.6	17.8	32.7	**16.5 ± 11.8**
	Cefazolin	4.1	4.4	5.6	16.5	**7.7 ± 6.0**
	Cefoperazone	12.5	9.0	8.6	20.5	**12.7 ± 5.5**
	Cefquinome	3.3	2.1	4.8	8.1	**4.6 ± 2.6**
Aminoglycosides	Kanamycin	6.2	4.2	3.4	17.8	**7.9 ± 6.7**
Folate Pathway Inhibitors	Trimethoprim-sulfamethoxazole	9.0	8.1	9.8	12.5	**9.9 ± 1.9**
Quinolones	Enrofloxacin	2.3	1.8	2.2	3.5	**2.5 ± 0.7**
	Danofloxacin	9.3	8.3	9.4	14.9	**10.5 ± 3.0**
Tetracyclines	Tetracycline	16.8	13.6	17.4	23.3	**17.8 ± 4.1**

a classification of antimicrobials to antimicrobial classes, according to Clinical and Laboratory Standards Institute (Table 4–9, 2012)

b Mean, standard deviation (± SD)

TABLE 9: *Occurrence (%) of antimicrobial resistant Enterococcus faecalis isolates.*

Antimicrobial class[a]	Antimicrobial agent	2011	2012	2013	2014	Mean ± SD[b]
Penicillins	Penicillin G	69.9	73.5	95.2	96.7	**83.8 ± 14.1**
	Ampicillin	84.7	64.7	73.0	69.9	**73.1 ± 8.5**
	Cloxacillin	87.7	81.0	94.0	93.7	**89.1 ± 6.2**
β-Lactam/β-Lactamase Inhibitor Combinations	Amoxicillin-clavulanic acid	1.2	1.1	0.8	1.7	**1.2 ± 0.4**
Cephems (including cephalosporins)	Cefalexine	74.2	59.5	74.7	70.6	**69.8 ± 7.1**
	Cefazolin	52.8	41.3	58.7	61.7	**53.6 ± 9.0**
	Cefoperazone	68.1	53.2	72.5	69.5	**65.8 ± 8.6**
	Cefquinome	31.3	19.3	35.7	30.1	**29.1 ± 7.0**
Macrolides	Tylosin	92.0	92.5	94.0	95.8	**93.6 ± 1.7**
Quinolones	Enrofloxacin	9.2	11.2	17.7	25.5	**15.9 ± 7.4**
Ansamycins	Rifaximin	77.9	82.5	82.4	80.8	**80.9 ± 2.1**

a classification of antimicrobials to antimicrobial classes, according to Clinical and Laboratory Standards Institute (Table 4–9, 2012)

b Mean, standard deviation (± SD)

TABLE 10: *Multi-resistance: Resistance (%) to three or more antibiotic agents.*

Pathogen	Year	Resistance ≥ three antibiotic agents (%)
S. aureus	2011	1.9
	2012	1.7
	2013	2.8
	2014	3.4
	Mean ± SDa	**2.4 ± 0.8**
S. uberis	2011	2.3
	2012	3.0
	2013	2.8
	2014	7.7
	Mean ± SDa	**4.0 ± 2.5**
NAS	2011	1.9
	2012	1.1
	2013	3.0
	2014	2.1
	Mean ± SDa	**2.0 ± 0.8**
S. dysgalactiae	2011	1.1
	2012	2.7
	2013	2.2
	2014	7.8
	Mean ± SDa	**3.4 ± 3.0**
E. coli	2011	15.3
	2012	12.9
	2013	15.5
	2014	41.8
	Mean ± SDa	**21.4 ± 13.7**
E. faecalis	2011	94.5
	2012	92.2
	2013	97.2
	2014	99.1
	Mean ± SDa	**95.8 ± 3.1**

a Mean, standard deviation (± SD)

associated with clinical and subclinical mastitis in several countries, such as Australia, New Zealand, Belgium and the United Kingdom (Davies et al. 2015, Wyder et al. 2011), showed a high prevalence over all four years.

On evaluating the proportion of pathogen-positive quarter milk samples per cow, we found that *E. coli*, *S. dysgalactiae* and *S. uberis* were mainly confined to only one of the four quarter samples per cow. Therefore, it is likely that these species have a lower tendency for spreading between udder quarters, and are thus possibly less contagious. *Staphylococcus aureus*, NAS and *E. faecalis* showed a higher potential for contagious spread. *Staphylococcus aureus* is widely considered a very contagious mastitis pathogen, spreading between quarters and animals (Sears and McCarthy 2003).

Depending on the causative pathogen, when infection occurred, its severity and rate of progression (peracute, acute, chronic, subclinical, clinical), bovine mastitis can be treated with antibiotics, either locally (intramammary), systemically or with a combined approach (McDougall et al. 2014, Rainard et al. 2017, Winter 2008). For treatment of mastitis caused by streptococci and staphylococci, penicillin G is still recommended as first choice antibiotic (Rüegsegger et al. 2014, Winter 2008).

Reassuringly, the susceptibility of *S. uberis* and *S. dysgalactiae* isolates to penicillin G was high. This result corroborates findings of studies in other countries, including Switzerland and the USA (Erskine et al. 2002, Rüegsegger et al. 2014). In contrast, for staphylococci, including *S. aureus* and non-aureus staphylococci, penicillin G resistance was about four times higher than for streptococci. For *S. aureus* especially, penicillin-resistance appears to be highly variable among countries as well as in different parts of individual countries (Aarestrup and Jensen 1998, Oliver and Murinda 2012). The antimicrobials of choice for mammary infections with penicillinase-producing staphylococci are macrolides (e. g. tylosin) and isoxazolyl penicillins, such as cloxacillin (Wendt et al. 1993, Winter 2008).

Particularly striking was the high resistance of Gram-positive cocci, especially *S. uberis*, against tylosin. Tylosin is labeled in Europe for the treatment of bovine mastitis and, as a macrolide, has a narrow spectrum of activity, which is primarily limited to Gram-positive bacteria (Bonnier et al. 2006). Further, the prevalence of tylosin resistant *S. aureus* was low and constant over four years in a study in France (Entorf et al. 2014), and therefore this antibiotic was still recommended for use against Gram-positive cocci in 2006 (Bonnier et al. 2006), especially as an alternative antibiotic for mastitis caused by penicillinase-producing staphylococci -as described above (Winter 2008). Entorf et al. (2014) compared the two macrolides tylosin and erythromycin against *S. aureus* mastitis isolates and discovered that almost 10% of isolates were resistant to tylosin, which is similar to our findings. However, despite the quality control parameters, there are still no approved clinical breakpoints available for this macrolide (Entorf et al. 2016), which makes it difficult to classify the bacteria into susceptible and resistant isolates.

The other examined Gram-positive cocci species, *E. faecalis*, was found to have high resistance against almost all antibiotics. This mastitis pathogen is generally described as resistant to many antibiotic agents (Wendt et al. 1993). Antibiotics associated with less than 50 % of resistant *E. faecalis* isolates were amoxicillin-clavulanic acid, enrofloxacin and cefquinome. However, since enterococci are animal environment (especially feces) pathogens, and mastitis caused by these bacteria is associated with poor hygiene (Wendt et al. 1993, Winter 2008), other preventive management strategies should be considered for enterococcal mastitis.

Less than 10% of Gram-negative *E. coli* were resistant to enrofloxacin, cefquinome, cefazolin and kanamycin. Systemic fluorquinolones and other broad-spectrum antibiotics, such as cephalosporins (ceftiofur and cefquinome), oxytetracycline, trimethoprim-sulfonamides, are effective for *E. coli* mastitis (Suojala et al. 2013, Winter 2008). It is important to point out that many of these antimicrobials, such as agents of the penicillin-, the 3rd and 4th generation cephalosporin- and the quinolone-class, are listed as critically important antimicrobials for human medicine and that therefore the use in veterinary medicine should be kept at an absolute minimum because of the high risk of spreading resistant bacteria from animals to humans (European Medicines Agency and European Food Safety Authority 2017, World Health Organization 2017). However, an adequate and fast treatment of sick animals is vital, and thus antimicrobial usage is often crucial. Therefore a prudent use of antimicrobials is essential. Moreover, the success of *E. coli* mastitis treatment depends on the severity of the disease. Intramammary treatment of *E. coli* mastitis in mild-to-moderate cases is not recommended (Suojala et al. 2013).

The number of isolates resistant to three or more antimicrobials was generally low. Interestingly, although the NAS group is generally assumed to exhibit high multi-resistance (Taponen and Pyörälä 2009), we found a lower percentage of multi-resistant NAS strains compared with *S. aureus* isolates. A high occurrence of multi-resistance was found with *E. coli.* Especially noticeable was the increase in multi-resistant *E. coli* isolates between 2011 and 2014. In 2014 more than twice as many multi-resistant isolates were detected. In addition, there was a high number of multi-resistant *E. faecalis* isolates. As mentioned above, *E. faecium* and *E. faecalis* are intrinsically resistant to many antibiotics, and resultantly they can cause serious infections (Hayes et al. 2004). Comparison of our findings with other studies is difficult since the term *multi-resistance* is often not clearly defined or simply applied if resistance is detected to several agents of the same antimicrobial class. Moreover, studies from other countries must be compared with caution due to the use of different licensed and tested veterinary antimicrobials.

Our results confirm that dairy farming in Austria is undergoing change. The herd size is increasing, while the number of farms is decreasing. The dominant bovine udder pathogens in quarter milk samples were mainly Gram-positive cocci, especially *S. aureus*, *S. uberis* and non-aureus staphylococci. However, the propensity of infections to spread among udder quarters, attributed to their contagious potential, and a trend suggestive of increased resistance to several antimicrobial agents, is pathogen-dependent. Further, an increased number of multi-resistant *E. coli* isolates was especially striking.

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Conflict of interest

The authors declare that they have no competing interests.

Ethics approval and consent to participate

Not applicable.

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Authors contribution

All raw data was provided by BL and GS (Upper Austrian TGD). Statistical analysis was performed by JH. Standard Operation Procedures (SOPs) were created and provided by BL. Results were interpreted and manuscript prepared by AS and KR. KR and MW coordinated the study. All authors read and approved the final manuscript.

Availability of data and materials

The datasets used and analysed during the current study are available from the corresponding author (KR), JH or from the Upper Austrian TGD (GS) upon reasonable request.

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