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ANTIMICROBIAL SUSCEPTIBILITY OF STREPTOCOCCI MOST FREQUENTLY ISOLATED FROM CZECH DAIRY COWS WITH MASTITIS*

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Abstract

The aim was to investigate the antimicrobial susceptibility of most frequently isolated streptococci from Czech dairy herds. A total of 3,719 quarter milk samples were collected and cultivated between January 2017 and June 2018 from cows with clinical or subclinical mastitis from 112 farms. Only one isolate of each species, collected from the same farm per six-month period, was included in the susceptibility testing. The susceptibilities of *Streptococcus uberis* (163 isolates) and *S. dysgalactiae* (25 isolates) to 10 antimicrobials (penicillin – PEN, amoxicillin/clavulanic acid – AMC, ceftiofur – EFT, clindamycin – CLI, gentamicin – GEN, streptomycin – STR, trimethoprim/sulfamethoxazole – SXT, enrofloxacin – ENR, tetracycline – TET, rifampicin – RIF) from 9 groups were determined by measuring their minimum inhibitory concentrations. The percentages of resistant *S. uberis* isolates to the antimicrobials were as follows: TET (63.2%), STR (52.1%), CLI (30.1%), and RIF (2.5%). Intermediate susceptibility was found to RIF (63.2%), PEN (35%), ENR (2.5%), EFT (1.8%), and AMC (1.2%). All the *S. uberis* isolates were susceptible to GEN and SXT (100%). However, only 6.7% of *S. uberis* isolates were susceptible to all tested antimicrobials, and 38.7% of isolates were multidrug resistant (≥ 3 groups of antimicrobials). All the *S. dysgalactiae* isolates were susceptible to PEN, AMC, EFT, GEN, SXT, and ENR (100%). Resistant *S. dysgalactiae* isolates were found to TET (60%), STR (28%), CLI (12%), and intermediate to TET (24%) and RIF (20%). Sixteen percent of *S. dysgalactiae* isolates were multidrug resistant. The relatively high occurrence of (multiple) resistance, relative to mastitis pathogens, highlights the importance of monitoring this condition in dairy herds.

Key words: cattle, *S. uberis*, *S. dysgalactiae*, MIC, multiple resistance

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In dairy herds, mastitis is one of the most frequently diagnosed diseases and causes significant losses to farmers (Halasa et al., 2007). The etiology of infectious mastitis involves numerous organisms as diverse as bacteria, mycoplasma, yeasts, and algae, but streptococci, staphylococci, and coliform bacteria (*Escherichia coli*) are considered to be the major mastitis pathogens (Watts, 1988; Bradley, 2002). Among gram-positive organisms, environmental streptococci, and coagulase-negative staphylococci (CNS) are currently the most prevalent pathogens recovered from clinical cases of mastitis in the USA, New Zealand, United Kingdom, and Switzerland (Bradley, 2002; McDougall et al., 2007; Oliveira et al., 2013; Rüegsegger et al., 2014). Similarly, in the Czech Republic, Bzdil (2012) showed that the most common mastitis pathogen was *Streptococcus* (*S.*) *uberis* (22.1%).

Mastitis is the most common reason for the use of antimicrobials in dairy cows, and they are an important part of mastitis treatment (de Jong et al., 2018). The antimicrobials used to treat intramammary infections in cows are similar across the world; however, the frequency of use differs between countries (Oliver and Murinda, 2012). In Europe (Belgium, Germany, France, Spain, Sweden, and the UK) the β-lactams (29–84%; 84% = Sweden) are commonly used, including penicillin, aminopenicillins (including combinations with clavulanic acid), isoxazolyl penicillins, and cephalosporins, particularly the 3rd and 4th generations. To a lesser extent, aminoglycosides and macrolides are also used (De Briyne et al., 2014). In the Czech Republic, the whole group of beta-lactams, e.g., amoxicillin in combination with a beta-lactamase inhibitor (e.g., clavulanic acid) are commonly used in the treatment of mastitis, together with 3rd and 4th generation cephalosporins (Nedbalcova et al., 2014).

Ideally, when deciding on an antimicrobial treatment for mastitis, the antimicrobial susceptibility of the udder pathogens should be known. However, since mastitis therapy is commonly initiated before pathogen susceptibility testing, monitoring antimicrobial resistance trends over time is very important (de Jong et al., 2018).

There are many non-European (McDougall et al., 2014; Ruegg et al., 2015; Cameron et al., 2016), European (Bengtsson et al., 2009; Burmańczuk et al., 2016; Crestani et al., 2016) and pan-European studies (Thomas et al., 2015; de Jong et al., 2018) dealing with the susceptibility of mastitis pathogens to antimicrobials. Over the past decade, several national programs have monitored the susceptibility of important veterinary pathogens to antimicrobials, e.g., Germany – GERMAP (2014) and Sweden – SVARM (SVA, 2018). A similar, but voluntary, national program has been functioning in the Czech Republic since 2015 (SVS ČR, 2017). The most disturbing fact revealed by the program was that 60.2% of mastitis pathogens in the Czech Republic carried at least one antibiotic resistance gene and 44.6% were multidrug-resistant (Pyatov et al., 2017). In the Czech Republic, only a few extensive studies of antimicrobial susceptibility of mastitis pathogens have been performed up to the present time.

The objective of this study was to investigate antimicrobial susceptibility of the most frequently isolated streptococci originating from Czech dairy cows with mastitis.

Material and methods

Milk samples

A total of 3,719 quarter milk samples were collected from cows with clinical or subclinical mastitis. The samples were delivered on a voluntary basis by local veterinarians from 112 farms in the eastern part of the Czech Republic, between January 1st, 2017 and June 30th, 2018, to pick-up points of the State Veterinary Institute, Olomouc, Czech Republic. The samples were transported in cool boxes at 4°C.

Isolation and identification of bacteria

The samples were subjected to conventional bacteriology (cultivation, isolation of the agents and their identification). All milk samples were inoculated onto Meat Peptone Blood Agar (MPBA) (Trios Ltd., Prague, Czech Republic) and incubated aerobically at 37 ± 1°C for 42–48 hours. In parallel, the same milk samples were placed in culture tubes with MPBA and incubated at 37 ± 1°C for 18–24 hours. Subsequently, the incubated milk samples were inoculated onto Edward´s Agar (Trios Ltd., Prague, Czech Republic). The inoculated Edward´s Agar plates were incubated at 37 ± 1°C for another 18–24 hours (Bzdil, 2004). On plates with mixed bacterial cultures, the most frequent colony forming agent was regarded as the major pathogen.

Suspicious colonies of streptococci were isolated. The isolated strains were subsequently confirmed using phenotypic molecular mass spectrometry, MALDI TOF MS, based on proteomics analyses, and MALDI Biotyper software (Bruker Daltonik GmbH, Bremen, Germany) (Štromerová, 2013).

Antimicrobial susceptibility testing

Selected isolates of the most frequently isolated streptococci were tested for susceptibility to a variety of antimicrobial agents. No more than one isolate of each species collected from the same farm per six-month period was included in antimicrobial susceptibility testing (AST). Isolates from animals that had been treated with antimicrobials during the two weeks prior to sampling were not included in the study. The minimum inhibitory concentrations (MICs) of antimicrobials were determined for 163 isolates of *S. uberis* and 25 isolates of *S. dysgalactiae.*

The resistance of isolates to antimicrobials was tested using a standardized microdilution method for determining MICs and followed international standardized methodologies. The MIC sets were prepared according to the method described in Clinical Laboratory Standard Institute (CLSI) document VET01-A4 (CLSI, 2013) and interpretation of the results was performed by clinical breakpoints published in CLSI supplements VET01S and VET08 (CLSI, 2015; CLSI, 2018), European Committee on Antimicrobial Susceptibility Testing – Breakpoint tables for bacteria (EUCAST, 2018) and Comité de l'Antibiogramme de la Société Francaise de Microbiologie – Recommandations vétérinaires 2018 (CA-SFM, 2018). The testing was performed using kits manufactured in the laboratory of the authors at the Veterinary Research Institute in Brno. The kits were manufactured using CLSI standard methods. According to CLSI documents, special microdilution trays were prepared.

These trays, with twofold dilutions of tested antimicrobial agents (Discovery Fine Chemicals, UK) in Mueller-Hinton Broth (Becton, Dickinson and Company, USA) with 4% Lysed Horse Blood (LabMediaServis, CR), were designed for antimicrobial susceptibility testing of veterinary pathogens and included various bacterial species. The tested antimicrobials were therefore selected from a wider spectrum of antimicrobials than those typically used in the treatment of mastitis, i.e., they included antimicrobials used to treat other bacterial diseases of farm animals; e.g., ceftiofur, a cephalosporin, was included in spite of the fact that it is not registered for the treatment of mastitis in the Czech Republic and the injectable form is not indicated for the treatment of mastitis due to its pharmacokinetic characteristics, nonetheless, it was the most frequently used cephalosporin in cattle (Nedbalcova et al., 2014). Quality control of the testing kits and the testing itself were performed using the *S. pneumoniae* ATCC 49619 reference strain. The tested antimicrobials and their respective concentration ranges are listed in Table 1.

Table 1. Tested antimicrobials and their concentrations

Antimicrobials	Tested concentrations (mg/L)
Penicillin	0.06–8
Amoxicillin/clavulanic acid (2/1)[a]	0.5–64
Ceftiofur	0.25–32
Clindamycin	0.125–16
Gentamicin	2–8 and 128–256
Streptomycin	2–32 and 256–1024
Trimethoprim/sulfamethoxazole (1/19)[b]	0.25–32
Enrofloxacin	0.06–8
Tetracycline	0.25–32
Rifampicin	0.03–4

[a] concentrations relative to amoxicillin.
[b] concentrations relative to trimethoprim.

The MICs values were read as the lowest concentration of an antimicrobial agent that inhibited visible bacterial growth. MIC_{50} and MIC_{90} are the lowest concentrations of antimicrobial substances, in mg/L, that inhibited the growth of 50% and 90% of isolates as determined by cumulative conversion (Schwarz et al., 2010). Since there are no interpretative AST criteria for mastitis pathogens for the majority of antimicrobials (except for ceftiofur (CLSI, 2018)), the categorizing of isolates as susceptible, intermediate, and resistant was performed according to human-derived clinical breakpoints (CLSI, 2015; CA-SFM, 2018; CLSI, 2018; EUCAST, 2018) – see Table 2.

For each pathogen, the profiles of phenotypic resistances from non-susceptible (resistant and intermediate) isolates to individual antimicrobials were assembled. Based on the profiles, multidrug resistance can be evaluated. A multidrug-resistant

isolate was defined as an isolate that was not sensitive to at least one agent in three or more antimicrobial groups (Magiorakos et al., 2011). The 10 antimicrobials used for testing represented 9 antimicrobial groups: lincosamides, aminoglycosides, sulfonamides, quinolones, tetracyclines, ansamycins; the penicillins (narrow spectrum, penicillinase sensitive), penicillins with beta-lactamase inhibitors (amoxicillin with clavulanic acid), and cephalosporins (3rd generation) were considered as three separate groups.

Table 2. Breakpoint table for *Streptococcus* spp.

Antimicrobials	MIC breakpoint (mg/L)			Source
	S	I	R	
Penicillin (*Streptococcus viridans* group – *S. uberis*)	≤ 0.25	0.5–2	≥ 4	EUCAST, 2018
Penicillin (β-hemolytic streptococci – *S. dysgalactiae*)	≤ 0.25	–	≥ 0.5	EUCAST, 2018
Amoxicillin/clavulanic acid (2/1)[a]	≤ 0.5	1–2	≥ 4	CLSI, 2015
Ceftiofur	≤ 2	4	≥ 8	CLSI, 2018
Clindamycin	≤ 0.5	–	≥ 1	EUCAST, 2018
Gentamicin	≤ 128	–	≥ 256	CLSI, 2015
Streptomycin	≤ 256	–	≥ 512	CA-SFM, 2018
Trimethoprim/sulfamethoxazole (1/19)[b]	≤ 0.5	1–2	≥ 4	CLSI, 2018
Enrofloxacin	≤ 0.5	1–2	≥ 4	CA-SFM, 2018
Tetracycline (*Streptococcus viridans* group – *S. uberis*)	≤ 2	4	≥ 8	EUCAST, 2018
Tetracycline (β-hemolytic streptococci – *S. dysgalactiae*)	≤ 1	2	≥ 4	EUCAST, 2018
Rifampicin	≤ 0.06	0.125–0.5	≥ 1	EUCAST, 2018

[a] concentrations relative to amoxicillin.
[b] concentrations relative to trimethoprim.
S – susceptible; I – intermediate; R – resistant.

Results

Potential mastitis pathogens were found in 47.1% of the milk samples, i.e., in 1,752 out of 3,719. A total of 2,284 isolates were gram-positive (G+) microorganisms, of which 708 (in 19.0% milk samples; 31.0% of G+ isolates) were *S. uberis*, *S. dysgalactiae*, and *S. agalactiae*. Samples with no growth or contamination (massive presence of more than 4 microbial species) constituted 21.4% and 31.5% of all samples, respectively. *S. uberis* (648 isolates) was the most frequently isolated pathogen (in 17.4% milk samples; 28.4% of G+ isolates); *S. dysgalactiae* with 41 isolates (1.1%; 1.8%); and *S. agalactiae* with 19 isolates (0.5%; 0.8%), was markedly less frequent.

Distributions of MICs for individual antimicrobials and species and MIC_{50} and MIC_{90} values are shown in Tables 3 and 4.

Table 3. Minimum inhibitory concentration (MIC) distribution for *Streptococcus uberis* isolates (n = 163) from dairy cows with mastitis

Mg/L	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024	2048	MIC50	MIC90
PEN		12	21	73	57													0.25	0.5
AMCa					161	2												≤0.5	≤0.5
EFT				54	8	42	56	3										1	2
CLI			105	7	2	1	7	36	3		2							≤0.125	4
GEN							5	5	24				129					≤128	≤128
STR									3	2	11			62	1	7	77	1024	>1024
SXTb				162	1													≤0.25	≤0.25
ENR		2	4	66	87	4												0.5	0.5
TET				56	1	2		1	3	24	29	47						16	>32
RIF	53	3	82	20	1			2	2									0.125	0.25

PEN – penicillin; AMC – amoxicillin/clavulanic acid; EFT – ceftiofur; CLI – clindamycin; GEN – gentamicin; STR – streptomycin; SXT – trimethoprim/sulfamethoxazole; ENR – enrofloxacin; TET – tetracycline; RIF – rifampicin.

[a]Amoxicillin and clavulanic acid in the ratio 2:1; test ranges are expressed as the amoxicillin concentration.

[b]Trimethoprim and sulfamethoxazole in the ratio 1:19; test ranges are expressed as the trimethoprim concentration: The dilution ranges tested are those contained within the white area. Values above this range indicate MIC values higher than the highest concentration in the range. Values corresponding to the lowest concentration tested indicated MIC values smaller or equal to the lowest concentration in the range. Breakpoints of resistance used are indicated with vertical black lines when available.

Table 4. Minimum inhibitory concentration (MIC) distribution for *Streptococcus dysgalactiae* isolates (n = 25) from dairy cows with mastitis

Mg/L	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024	2048	MIC50	MIC90
PEN		25																≤0.06	≤0.06
AMCa					25													≤0.5	≤0.5
EFT				25														≤0.25	≤0.25
CLI			19	3							3							≤0.125	>16
GEN								2	15				8					8	≤128
STR										13	4			1	4	3		16	1024
SXTb				25														≤0.25	≤0.25
ENR				6	19													0.5	0.5
TET				1		3	6	2			8	5						32	>32
RIF	8	12	5															0.06	0.125

PEN – penicillin; AMC – amoxicillin/clavulanic acid; EFT – ceftiofur; CLI – clindamycin; GEN – gentamicin; STR – streptomycin; SXT – trimethoprim/sulfamethoxazole; ENR – enrofloxacin; TET – tetracycline; RIF – rifampicin.

[a]Amoxicillin and clavulanic acid in the ratio 2:1; test ranges are expressed as the amoxicillin concentration.

[b]Trimethoprim and sulfamethoxazole in the ratio 1:19; test ranges are expressed as the trimethoprim concentration: The dilution ranges tested are those contained within the white area. Values above this range indicate MIC values higher than the highest concentration in the range. Values corresponding to the lowest concentration tested indicated MIC values smaller or equal to the lowest concentration in the range. Breakpoints of resistance used are indicated with vertical black lines when available.

Isolates of each bacterial species were categorized as susceptible, intermediate, or resistant using clinical breakpoints (Table 2). Percentages of isolates susceptible, intermediate, or resistant to tested antimicrobials are shown in Figures 1 and 2. The profiles of phenotypic resistances are shown in Tables 5 and 6.

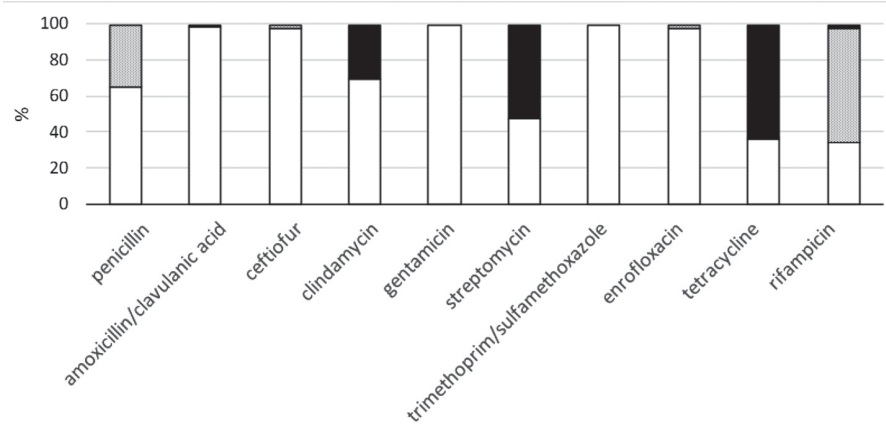

Figure 1. Percentages of *S. uberis* isolates (n = 163) susceptible, intermediate, or resistant to tested antimicrobials

Table 5. Resistance profiles for *Streptococcus uberis* isolates (n=163)

Frequency of resistance by		Phenotype of resistance	Number of resistant isolates	Number of multidrug-resistant isolates
active substance	antimicrobial groups			
1	2	3	4	5
0	0		11 (6.7%)	100 (61.3%)
1	1	RIF	30 (18.4%)	
1	1	TET	12 (7.4%)	
1	1	STR	4 (2.5%)	
1	1	ENR	1 (0.6%)	
1	1	EFT	1 (0.6%)	
2	2	TET, RIF	19 (11.7%)	
2	2	STR, TET	14 (8.6%)	
2	2	STR, RIF	4 (2.5%)	
2	2	PEN, TET	2 (1.2%)	
2	2	CLI, RIF	1 (0.6%)	
2	2	PEN, RIF	1 (0.6%)	

Table 5 – contd.

1	2	3	4	5	6
3	3	STR, TET, RIF	11	(6.7%)	
3	3	CLI, STR, TET	7	(4.3%)	
3	3	CLI, STR, RIF	7	(4.3%)	
3	3	ENR, TET, RIF	1	(0.6%)	
3	3	PEN, STR, TET	1	(0.6%)	
3	3	CLI, TET, RIF	1	(0.6%)	
4	4	CLI, STR, TET, RIF	25	(15.3%)	63 (38.7%)
4	4	PEN, STR, TET, RIF	1	(0.6%)	
4	4	EFT, STR, TET, RIF	1	(0.6%)	
4	4	CLI, STR, ENR, TET	1	(0.6%)	
5	5	PEN, CLI, STR, TET, RIF	6	(3.7%)	
7	7	PEN, EFT, CLI, STR, ENR, TET, RIF	1	(0.6%)	

PEN – penicillin; EFT – ceftiofur; CLI – clindamycin; STR – streptomycin; ENR – enrofloxacin; TET – tetracycline; RIF – rifampicin.

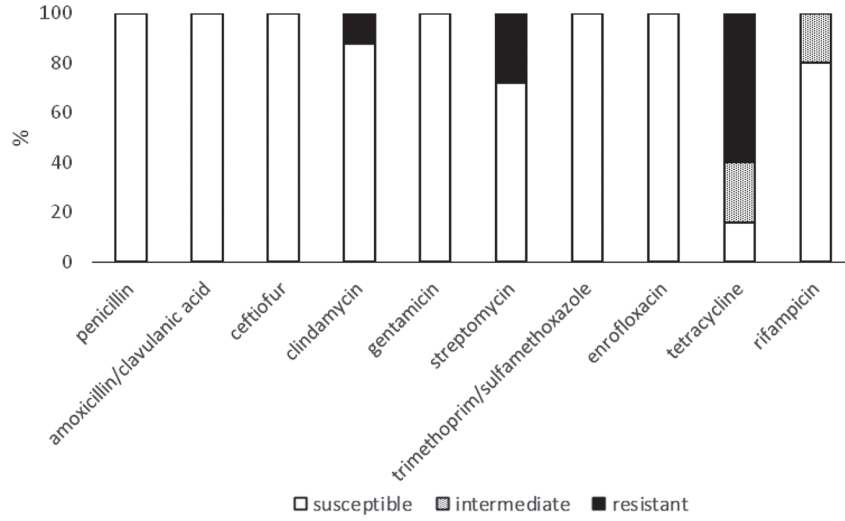

Figure 2. Percentages of *S. dysgalactiae* isolates (n=25) susceptible, intermediate, or resistant to tested antimicrobials

The highest percentage of resistant *S. uberis* isolates was found to tetracycline (63.2%), and streptomycin (52.1%), with a significant percentage of *S. uberis* isolates also being resistant to clindamycin (30.1%). On the other hand, all the isolates tested were susceptible to gentamicin and trimethoprim/sulfamethoxazole. Most of the tested isolates were susceptible to amoxicillin/clavulanic acid (98.8%), ceftiofur

(98.2%), and enrofloxacin (97.5%). The isolates were categorized as being intermediate relative to rifampicin (63.2%) and penicillin (35.0%). However, none or only a few isolates were resistant to penicillin (0%) and rifampicin (2.5%).

Only 11 (6.7%) *S. uberis* isolates were found to be susceptible to all tested antimicrobials and quite a lot were multidrug resistant (63 isolates, 38.7%). The most frequent combination of resistances was to clindamycin, streptomycin, tetracycline, and rifampicin, in 25 (15.3%) isolates.

All the *S. dysgalactiae* isolates were susceptible to penicillin, amoxicillin/clavulanic acid, ceftiofur, gentamicin, trimethoprim/sulfamethoxazole, and enrofloxacin. In terms of resistance, *S. dysgalactiae* isolates were resistant to tetracycline (60.0%), streptomycin (28.0%), and clindamycin (12.0%). Additionally, *S. dysgalactiae* isolates were categorized as intermediate relative to tetracycline (24.0%) and rifampicin (20.0%).

Table 6. Resistance profiles for *Streptococcus dysgalactiae* isolates (n = 25)

Frequency of resistance by		Phenotype of resistance	Number of resistant isolates		Number of multidrug-resistant isolates
active substance	antimicrobial groups				
0	0		2	(8%)	0
1	1	TET	12	(48%)	
1	1	STR	1	(4%)	
1	1	RIF	1	(4%)	
2	2	TET, RIF	3	(12%)	
2	2	STR, TET	2	(8%)	
3	3	CLI, STR, TET	3	(12%)	4 (16%)
3	3	STR, TET, RIF	1	(4%)	

CLI – clindamycin; STR – streptomycin; TET – tetracycline; RIF – rifampicin.

In contrast to *S. uberis*, only 4 isolates of *S. dysgalactiae* were defined as multiresistant (16%). However, the results of antimicrobial susceptibility testing obtained with these pathogens should be assessed cautiously because of the disproportionate number of *S. uberis* (n = 163) isolates compared to *S. dysgalactiae* (n = 25) isolates tested.

Discussion

This study is one of the first to deal with antimicrobial sensitivity of mastitis pathogens in Czech dairy herds. The largest proportion of antimicrobial use in dairy cows in Europe is attributable to udder health (De Briyne et al., 2014) and although the administration is mainly intramammary and not systemic, their use presents a potential risk of antimicrobial resistance.

Many of these drugs are also widely used in human medicine, and their routine use in veterinary medicine poses a potential problem. There is a concern that the veterinary use of these drugs could lead to the selection and development of resistant bacteria, which can be dangerous to human health (McEwen and Fedorka-Cray, 2002).

With a 17.4% prevalence, *S. uberis* was the most frequently isolated mastitis pathogen in other studies (Kalmus et al., 2011; Rüegsegger et al., 2014; Supré et al., 2014) as well as studies from the Czech Republic (Bzdil, 2012). *S. uberis* was also the most frequently tested mastitis pathogen in the Czech national antibiotic program (SVS ČR, 2017).

There are many international papers dealing with antimicrobial susceptibility of pathogenic streptococci recovered from clinical and subclinical mastitis cases (Guérin-Faublée et al., 2002; Pitkälä et al., 2004; Kalmus et al., 2011; Cameron et al., 2016; Kaczorek et al., 2017). They differ in some respects such as the method of isolate collection, selection of tested antimicrobials, and method of susceptibility testing. The most important criterion for further use of data is related to the interpretation of the results by presenting either summarized results, the frequency of MIC distribution, or susceptibility data based on different national or international breakpoints (Thomas et al., 2015). Large variability between studies makes data comparisons difficult. Furthermore, the differences between the results of antibiotic susceptibility reports from various regions of the world are very often closely related to the consumption of antimicrobials. There are several studies regarding the dependence of antimicrobial resistance on antimicrobial consumption (McEwen and Fedorka-Cray, 2002; Chantziaras et al., 2014; Nedbalcova et al., 2014; EPRUMA, 2017). Therefore, national antibiotic programs in different countries can have a great impact on the occurrence and spread of resistant isolates. All these facts must be considered when studying or comparing results from these studies.

Susceptibility ≥ 95% of streptococci to beta-lactams (penicillin and/or amoxicillin with clavulanic acid) presented in earlier studies (Guérin-Faublée et al., 2003; Bengtsson et al., 2009; Persson et al., 2011; Rüegsegger et al., 2014; Petrovski et al., 2015; de Jong et al., 2018) applies only to *S. dysgalactiae* isolates in our study. In *S. uberis,* we found only a 65% susceptibility to penicillin. The MIC_{50} and MIC_{90} values of 0.25 µg/mL and 0.5 µg/mL, respectively, found in this study were higher than those reported in other studies. Guérin-Faublée et al. (2003) reported an MIC_{50} of 0.03 µg/mL and an MIC_{90} of 0.25 µg/mL, Pol and Ruegg (2007) reported 0.12 µg/mL and Pitkälä et al. (2004) 0.06 µg/mL for both MIC_{50} and MIC_{90}. The high value (35%) of *S. uberis* isolates with intermediate susceptibility to penicillin found in our study differs from an 89% susceptibility of *S. uberis* found predominantly in parts of the Czech Republic within the framework of the voluntary National program of monitoring antimicrobial resistance of pathogens with veterinary importance (SVÚ Jihlava, 2018). However, our result is comparable to results from New Zealand (McDougall et al., 2014) and across Europe (Thomas et al., 2015) where approximately 30% of the analyzed isolates were classified as intermediate susceptible to the active substance. The isolation of such pathogens could be due to a mutation of penicillin-binding proteins, resulting in a decreased affinity for the drug (Haenni et al., 2010).

A high percentage of intermediately susceptible strains can present a risk that these pathogens will not respond well to *in vivo* therapy or may potentially evolve into resistant strains. Increased exposure of animals to antimicrobials or inappropriate dosing protocols (e.g., low doses administered for too short or too long a period) should be considered a significant risk factor for increased bacterial resistance in veterinary medicine (Lees et al., 2008).

According to many researchers, phenotypic resistance to tetracycline is the most common form of resistance in *Streptococcus* species (Guérin-Faublée et al., 2002; Bengtsson et al., 2009; Ruegg et al., 2015; Crestani et al., 2016; Kaczorek et al., 2017). Our study appears to confirm this; we found the lowest susceptibility and a very high frequency of resistance to tetracycline in *S. uberis* (63.2%) and *S. dysgalactiae* (60.0%) isolates. A similarly high occurrence of resistance (*S. uberis* (62.1%) and *S. dysgalactiae* (63.5%)) in the Czech Republic was found in a study monitoring antimicrobial susceptibility between 2015 and 2016 (SVS ČR, 2017). On the other hand, low resistance to tetracycline (12%) was reported in Sweden (Persson et al., 2011), where the use of tetracycline is significantly less than narrow-spectrum penicillins (De Briyne et al., 2014). The high resistance seen to tetracycline is probably because tetracyclines have been widely used, for many years, to treat a variety of ruminants infections (Kalmus et al., 2011), which is also true in the Czech Republic. It has been shown that many genetic determinants of tetracycline resistance can be actively transferred between bacterial genera and between hosts, both human and animal; as a result, resistance to tetracycline is found in almost all bacterial genera (Aminov et al., 2001).

Because of the intrinsic resistance of streptococci to aminoglycosides (streptomycin), which is a consequence of limited permeability of these antibiotics through the cell wall (Kaczorek et al., 2017), *S. uberis* isolates were seen to have low susceptibility to streptomycin (47.9%). Similarly, a low level of susceptibility to streptomycin was confirmed in Croatia with 54.3% (Leskovec et al., 2015); isolates from the USA and New Zealand (Petrovski et al., 2015) had an even lower susceptibility (2.2%).

In the Czech Republic, in addition to amoxicillin products, pirlimycin, linked to lincosamides has recently become popular for the treatment of *S. uberis*-caused mastitis. In our study, we did not directly investigate susceptibility to pirlimycin. Nevertheless, the relatively high resistance (30.1%) of *S. uberis* isolates to clindamycin (an antimicrobial used as a class representative when testing lincosamides, e.g., pirlimycin and lincomycin) can pose a certain risk. Similarly, it was reported (Pol and Ruegg, 2007) that in the USA, 24% of *Streptococcus* spp. were resistant to pirlimycin. From this point of view, we must be vigilant against the occurrence of resistance to pirlimycin and test the susceptibility of isolates to this antibiotic.

Despite the similarities discussed above, some differences in the frequency of resistant and intermediate isolates between the two main streptococcal species (*S. uberis* and *S. dysgalactiae*) were found. These were similar to findings reported by Petrovski et al. (2015) and Cameron et al. (2016). Our results demonstrated that most antimicrobials had better *in vitro* efficacy against *S. dysgalactiae* compared to *S. uberis* (e.g., penicillin, clindamycin, streptomycin, and rifampicin), whereas fewer

isolates of *S. dysgalactiae* were susceptible to tetracycline. However, we should consider the great disparity between the numbers of tested *S. uberis* (n = 163) and *S. dysgalactiae* (n = 25) isolates. Based on all our findings, it is advisable to identify pathogens on the species level, rather than the genus level, in order to recommend the best treatment for the respective farm/individual animals.

In our study, multidrug resistance was found in 35.6% (67/188) of the *Streptococcus* spp. isolates, which was very similar to the results (34.1%) from France (Guérin-Faublée et al., 2002), while Chinese researchers reported that 88.9% of their streptococci isolates (*S. agalactiae*, *S. uberis*, and *S. dysgalactiae*) were resistant to three or more antimicrobial groups (Ding et al., 2016).

In conclusion, *Streptococcus* spp. are important mammary gland pathogens, and in our study, they represented 31.1% of the gram-positive pathogens, the most frequent being *Streptococcus uberis,* which represented 28.4% of the gram-positive isolates. A very positive finding was that *S. uberis* and *S. dysgalactiae* were both highly sensitive to amoxicillin-clavulanic acid, trimethoprim-sulfamethoxazole as well as other critically important antimicrobials, under a prudent use regimen, such as ceftiofur, gentamicin, and enrofloxacin. On the other hand, a large percentage of *S. uberis* were found resistant to tetracycline, streptomycin, and clindamycin. Intermediate susceptibility to rifampicin and penicillin also poses a potential risk of an inadequate therapeutic response. Because of the substantial variation in susceptibility of streptococci, isolated from mastitis cases to tested antimicrobials, it is necessary to use antimicrobials for the treatment of mastitis in a prudent and targeted manner since there are significant differences in the spectra of causative agents and since susceptibilities vary among countries, farms, and investigative periods. These differences also depend on the use of different groups of antimicrobials for the treatment and prevention of all health problems. This is influenced not only by the attitude of veterinarians and farmers but also by national antibiotic policies and by portfolios of authorized and available veterinary products on the respective market. Sensible use of antimicrobials is only one of the building blocks of comprehensive and systematic mastitis control programs and should be used in conjunction with complex dairy herd management, preventive medicine, and biosecurity approaches to keep animals healthy.

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