

Determination of the minimum inhibitory concentration (MIC) and mutant prevention concentration (MPC) of selected antimicrobials in bovine and swine *Pasteurella multocida*, *Escherichia coli*, and *Staphylococcus aureus* isolates

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Abstract

We compared the values of the minimum inhibitory concentration (MIC) and mutant prevention concentration (MPC) values of three antimicrobial agents for 72 bovine isolates of *Pasteurella multocida*, 80 swine isolates of *P. multocida*, 80 bovine isolates of *Escherichia coli*, 80 swine isolates of *E. coli*, and 80 isolates of *Staphylococcus aureus* from bovine mastitis. The ratio of MIC_{90}/MPC_{90} which limited mutant selection window (MSW) was $\leq 0.12/4$ mg/l for enrofloxacin, $0.5/\geq 64$ mg/l for florfenicol and $4/\geq 128$ mg/l for tulathromycin in bovine *P. multocida* isolates, $\leq 0.12/2$ mg/l for enrofloxacin, $0.5/\geq 64$ mg/l for florfenicol and $4/\geq 128$ mg/l for tulathromycin in swine *P. multocida* isolates, $1/16$ mg/l for enrofloxacin, $8/\geq 64$ mg/l for florfenicol and $8/\geq 128$ mg/l for tulathromycin in bovine *E. coli* isolates, $0.5/16$ mg/l for enrofloxacin, $\geq 64/\geq 64$ mg/l for florfenicol and $8/\geq 128$ mg/l for tulathromycin in swine *E. coli* isolates, and $0.25/16$ mg/l for enrofloxacin, $4/\geq 64$ mg/l for florfenicol and $4/\geq 128$ mg/l for tulathromycin in *S. aureus* isolates. These findings indicate that the dosage of antimicrobial agents to achieve serum concentration equal to or higher than MPC could reduce selection of resistant bacterial subpopulation.

Bacteria, antibiotics, resistance, MIC, MPC, MSW, cattle, pigs

Effective antimicrobial therapy is compromised by spreading bacterial resistance which has been detected worldwide at different levels also in breeding animals. The reduction of bacterial resistance over time is mostly associated with a reduction in the consumption of antimicrobials (Aarestrup et al. 2008). However, there are a number of bacteria with mutations that prevent the action of antimicrobial substances and cause the selection of a resistant mutant during clinical treatment in the bacterial population susceptible to antimicrobials (Canton and Morosini 2011).

The antimicrobial agents are used for therapy on the basis of the dosage strategy for antimicrobials according to their pharmacokinetic (PK) and pharmacodynamic (PD) properties and determination of minimum inhibitory concentration (MIC) for the pathogen. A new concept in the testing of antimicrobial resistance with the potential to reduce bacterial resistance is the dosing of antimicrobials based on their PK/PD properties and determination of the mutant prevention concentration (MPC) for the pathogen (Drlica 2001). The MPC is defined as the concentration of antimicrobial substances able to prevent the growth of resistant mutants with a density of inoculum $\geq 10^9$ CFU/ml. The MPC is an indicator of susceptibility designed for a more accurate assessment of the potential of selection of a resistant mutant to antimicrobial substances than traditional methods for the determination of susceptibility (Zhao and Drlica 2008). It mostly better corresponds to the concentration of bacteria in the blood or in the target organ during acute infection of animals and people than MIC determination for inoculum with a density of 10^5 CFU/ml (Croisier et al. 2004; Zhao and Drlica 2008). The concentration range between the MIC and the MPC is called the mutant selection window (MSW). When this window is wider, the risk of selection of resistant strains is also greater (Drlica 2001; Tam et al. 2005; Blondeau 2009).

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The objective of this study was to compare the MIC and MPC values for enrofloxacin, florfenicol, and tulathromycin in *E. coli*, *P. multocida*, and *S. aureus* isolated from clinical cases of bovine or swine infections in the Czech Republic during the period from 2008 to 2012.

Material and Methods

Isolates

Isolates of enterotoxigenic *E. coli* were obtained from enteric infections of post-weaned calves (80 isolates) and piglets (80 isolates) with diarrhoea, *P. multocida* isolates from the lungs after necropsy of fattening cattle (72 isolates) and nursery or grower-finisher pigs (80 isolates) with respiratory disorders, and 80 *S. aureus* isolates from milk of dairy cows suffering from mastitis over the period 2008–2012. All *E. coli* isolates were positive in testing for the presence of the gene coding the production of enterotoxin STa, according to Cheng et al. (2006). *Pasteurella multocida* isolates were identified by PCR (Townsend et al. 1998) a *S. aureus* isolates were identified biochemically using the commercial kit STAPHYTEST (Erba Lachema, Czech Republic). The samples originated from animals without previous antimicrobial therapy during the last 3 weeks and the isolates from the same herds were included in the monitoring of MICs and MPCs of antimicrobials, with a minimum interval of 6 months from the last collection.

MIC, MPC and MSW determination

Susceptibility to antimicrobial substances was determined by assignment of MIC according to the standardised dilution micromethod of the Clinical and Laboratory Standards Institute (CLSI 2013a; 2013b) using commercial kits (Trek Diagnostics Systems Inc., England). The kit quality was controlled by the reference strains of *E. coli* ATCC 25922 and *S. aureus* ATCC 29213. The MIC values were defined as the lowest concentration of an antimicrobial agent inhibiting visible bacterial growth of culture with the density of 10^5 CFU/ml. Breakpoints of resistances were defined according to the CLSI and European Committee on Antimicrobial Susceptibility Testing (EUCAST 2013).

Determination of the MPC was made in duplicate by the dilution method according to CLSI (2013a; 2013b), using a modification of the previously published method (Blondeau 2001; Randall et al. 2004). Quality control of the experiment was performed with the reference strains of *E. coli* ATCC 25922 and *S. aureus* ATCC 29213. The MPC was determined as the lowest concentration of an antimicrobial agent inhibiting visible growth of bacterial culture with density of 10^9 CFU/ml.

The MSW was determined as the ranges of concentration between MPC_{90} and MIC_{90} .

Results

Distribution of MICs for enrofloxacin, florfenicol, and tulathromycin in bovine and swine *P. multocida* isolates, bovine and swine *E. coli* isolates, and *S. aureus* isolates from dairy cows mastitis and calculation of the MIC_{50} and MIC_{90} values are in Table 1. There are also comparative MPC distribution data for the isolates tested against the 3 antimicrobial agents and calculation of the MPC_{50} and MPC_{90} values. Table 2 shows the percentages of susceptible, intermediately susceptible, and resistant *P. multocida*, *E. coli*, and *S. aureus* isolates to enrofloxacin, florfenicol, and tulathromycin. The ranges between MIC_{90} and MPC_{90} that present the MSW for enrofloxacin, florfenicol, and tulathromycin are also shown.

As detected by the CLSI (2013a; 2013b) and EUCAST (2013) methods for MIC determination the majority of the tested bovine and swine *P. multocida* isolates were susceptible to the tested antimicrobial substances with MIC_{90} values below the breakpoint of resistance. On the other hand, bovine and swine *E. coli* isolates and *S. aureus* isolates from dairy cow mastitis were mostly resistant or intermediately susceptible to florfenicol. The MIC_{90} values for all three antimicrobials were the same in bovine and swine *P. multocida* isolates and *S. aureus* isolates, but differences were found between bovine and swine *E. coli*, except for tulathromycin. The MIC_{90} value for enrofloxacin was slightly higher in bovine *E. coli* isolates (bovine isolates 1 mg/l and swine isolates 0.5 mg/l) and marked difference (bovine isolates 8 mg/l and swine isolates ≥ 64 mg/l) was detected for florfenicol. The MPC values were above the susceptibility/resistance breakpoints of enrofloxacin, florfenicol, and tulathromycin in all cases. Even for florfenicol and tulathromycin, the MPC_{50} values

Table 1. MIC and MPC values of tested antimicrobials in respective isolates.

Isolates No. of isolates	Antimicrobials	MIC/MPC distribution values (mg/l)															MIC ₅₀ /MIC ₉₀	MPC ₅₀ /MPC ₉₀
		0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024	2048		
Bovine <i>P. multocida</i> 72	Enrofloxacin	68/1	0/2	2/15	0/27	1/15	1/10	0/1	0/0	0/1							≤0.12/≤0.12	1/4
	Florfenicol		27/0	42/0	1/0	1/1	0/1	1/2	0/12	0/47	0/9						0.5/0.5	32/≥64
	Tulathromycin			4/0	19/1	36/0	7/0	4/0	2/6	0/18	0/19	0/28					2/4	64/≥128
Swine <i>P. multocida</i> 80	Enrofloxacin	79/0	0/4	0/12	1/35	0/24	0/2	0/0	0/3								≤0.12/≤0.12	1/2
	Florfenicol		21/0	57/0	1/0	1/0	0/0	0/4	0/13	0/57	0/6						0.5/0.5	32/≥64
	Tulathromycin			2/0	24/0	33/0	16/0	5/0	0/2	0/11	0/45	0/22					2/4	64/≥128
Bovine <i>E. coli</i> 80	Enrofloxacin	70/0	1/1	5/6	0/34	0/27	0/3	0/1	0/1	4/7							≤0.12/1	2/16
	Florfenicol		0/0	0/0	1/0	5/0	19/0	50/0	1/0	0/0	4/80						8/8	≥64/≥64
	Tulathromycin			0/0	0/0	0/0	4/0	31/0	39/0	6/0	0/13	0/67					8/8	≥128/≥128
Swine <i>E. coli</i> 80	Enrofloxacin	69/0	5/4	3/15	0/33	0/7	1/9	1/7	0/0	1/5							≤0.12/0.5	2/16
	Florfenicol		0/0	0/0	0/0	2/0	26/0	32/0	9/0	1/0	10/80						8/≥64	≥64/≥64
	Tulathromycin			0/0	0/0	1/0	5/0	51/0	18/1	1/1	1/15	0/63					4/8	≥128/≥128
<i>S. aureus</i> 80	Enrofloxacin	67/0	10/0	2/0	2/2	25/15	1/42	0/16	0/4	0/1							≤0.12/0.25	8/16
	Florfenicol		0/0	0/0	2/0	25/0	50/0	2/1	1/0	0/0	0/79						4/4	≥64/≥64
	Tulathromycin			0/0	0/0	4/0	60/0	15/0	0/0	1/6	0/39	0/35					2/4	64/≥128

The dilution ranges tested are shown in the white area. Values above these ranges indicate MIC or MPC values higher than the highest concentration in the range. Values corresponding to the lowest concentration tested indicate MIC or MPC values less than or equal to the lowest concentration in the range.

P. multocida - Pasteurella multocida; E. coli - Escherichia coli; S. aureus - Staphylococcus aureus

The MIC₅₀ and MIC₉₀ values represent the lowest concentration (mg/l) inhibiting the growth of 50% and 90% of the tested isolates in the bacterial culture with a density of 10⁸ CFU/ml.

The MPC₅₀ and MPC₉₀ values represent the lowest concentration (mg/l) inhibiting the growth of 50% and 90% of the tested isolates in the bacterial culture with a density of 10⁸ CFU/ml.

in all *E. coli* and *S. aureus* isolates and the MPC₉₀ values in all tested isolates were above the range of concentrations.

We found quite wide ranges of concentrations between MIC₉₀ and MPC₉₀ (MSW) (Table 2). In most cases, they included the whole range of antimicrobials from the lowest tested concentrations under breakpoints of resistance to very high concentrations of antimicrobial agents, most often

Table 2. Susceptibility/resistance of isolates to tested antimicrobials and the ranges of MSW.

No. of isolates	Antimicrobials	MIC		MPC		R %	MIC ₅₀ /MIC ₉₀ (mg/l)	MPC ₅₀ /MPC ₉₀ (mg/l)	MSW MIC ₉₀ - MP (mg/l)
		S %	I %	S %	I %				
Bovine <i>P. multocida</i> 72	Enrofloxacin	97.2	1.4	1.4	18.3	16.7	$\leq 0.12 \leq 0.12$	1/4	$\leq 0.12 - 4$
	Florfenicol	98.6	0	1.4	1.4	97.2	0.5/0.5	32 \geq 64	0.5 - \geq 64
	Tulathromycin	100	0	0	25.0	65.3	2/4	64 \geq 128	4 - \geq 128
Swine <i>P. multocida</i> 80	Enrofloxacin	98.8	1.3	0	73.8	6.3	$\leq 0.12 \leq 0.12$	1/2	$\leq 0.12 - 2$
	Florfenicol	100	0	0	0	100	0.5/0.5	32 \geq 64	0.5 - \geq 64
	Tulathromycin	100	0	0	13.8	83.8	2/4	64 \geq 128	4 - \geq 128
Bovine <i>E. coli</i> 80	Enrofloxacin	95.0	0	5.0	76.3	15.0	$\leq 0.12/1$	2/16	1 - 16
	Florfenicol	7.5	23.8	68.8	0	100	8/8	$\geq 64 \geq 64$	8 - \geq 64
	Tulathromycin	92.5	7.5	0	0	100	8/8	$\geq 128 \geq 128$	8 - \geq 128
Swine <i>E. coli</i> 80	Enrofloxacin	96.3	0	3.8	50.0	26.3	$\leq 0.12/0.5$	2/16	0.5 - 16
	Florfenicol	2.5	32.5	65.0	0	100	8 \geq 64	$\geq 64 \geq 64$	$\geq 64 - \geq 64$
	Tulathromycin	93.8	5.0	1.3	1.3	97.5	4/8	$\geq 128 \geq 128$	8 - \geq 128
<i>S. aureus</i> 80	Enrofloxacin	98.8	0	1.3	21.3	78.8	$\leq 0.12/0.25$	8/16	0.25 - 16
	Florfenicol	33.8	62.5	3.8	0	100	4/4	$\geq 64 \geq 64$	4 - \geq 64
	Tulathromycin	98.8	1.3	0	7.5	92.5	2/4	64 \geq 128	4 - \geq 128

S (%) is the percentage of susceptible isolates

I (%) is the percentage of intermediately susceptible isolates

R (%) is the percentage of resistant isolates.

P. multocida - *Pasteurella multocida*; *E. coli* - *Escherichia coli*; *S. aureus* - *Staphylococcus aureus*

The MIC₉₀ value represents the lowest concentration (mg/l) inhibiting the growth of 90% of the tested isolates in the bacterial culture with a density of 10⁵ CFU/ml

The MPC₉₀ value represents the lowest concentration (mg/l) inhibiting the growth of 90% of the tested isolates in the bacterial culture with a density of 10⁹ CFU/ml

exceeding the highest tested concentration. The widest MSW were found in *P. multocida* isolates for florfenicol (0.5 - \geq 64 mg/l in bovine and swine isolates). It was possible to specify the width of MSW only for enrofloxacin where the values of MPC₉₀ were within the tested ranges of concentration, but unfortunately, all MPC₉₀ values were \geq 64 mg/l for florfenicol and \geq 128 mg/l for tulathromycin. Nevertheless, these not exactly defined values also represent the risk of the

selection of resistance, because the MICs values of tested isolates (low limiting concentration of MSW) were low and the isolates were susceptible to the tested antimicrobials, except for florfenicol (all *E. coli* isolates) and enrofloxacin (swine *E. coli* isolates).

Discussion

To establish effective risk management measures, objective and valid data on the development, selection and spread of resistance in bacteria are essential. The data collected in veterinary monitoring studies should allow us to (1) recognize the emergence of new resistance phenotypes, (2) determine time-dependent trends in the development of resistance, (3) deduce epidemiological correlation regarding the present situation, (4) assess the development and spread of antimicrobial resistance in relation to antimicrobial usage, and thereby (5) to estimate the risk in veterinary medicine. With regard to the possible impact on human health, it is important to assess the risk of antimicrobial resistance caused by the use of antimicrobials in veterinary medicine (Wallmann 2006). The susceptibility or resistance of isolates are determined by using the standardized methods (CLSI 2013a; 2013b; EUCAST 2013), among them being the MIC determination. However, in recent studies, some authors have also recommended MPC determination and its comparison with MIC for prediction of selection and spread of antimicrobial resistances (Hesje et al. 2007; Lees et al. 2008; Blondeau 2009).

This manuscript provides information about testing the MIC and MPC in clinical isolates of some bovine and swine pathogens for selected veterinary antimicrobials. In all cases, the MPC values were much greater than the value of the MIC that was found in the majority of isolates. These results are in accordance with previous studies of the spread of resistance to antimicrobial agents due to selective pressure during clinical treatment (Hesje et al. 2007; Blondeau 2009). This is because the growth of susceptible bacterial populations is inhibited by antimicrobials. However, the therapeutic dose is not able to prevent further reproduction of mutant bacteria (Lees et al. 2008; Blondeau 2009). The MPC values of antimicrobials can be used clinically to reduce the development of resistance by different dosing strategies together with the assessment of other PK/PD indicators. Information on certain pharmaceuticals is available also in package leaflets (the value, the speed of achieving the maximum concentration of antimicrobial substances, and the dynamics of its decrease in serum and different tissues). In order to avoid the spread of a resistant mutant in a bacterial population, the concentration of antimicrobial substances in serum should reach the MPC value provided that it is safe for the treated animal. These values may vary between different species of animals (Blondeau 2009).

When we compare the information on PK/PD properties of the tested antimicrobials (available on the drug manufacturer's website), the detected MPC₉₀ values are higher than the highest concentration reached at the infection site after administration of the recommended dose of antimicrobials for treatment (C_{max}). The C_{max} of enrofloxacin is 0.49 mg/l after administration of a dose of 2.5 mg/kg for cattle and 1.19 mg/l after the same dose for pigs. The C_{max} of florfenicol is 3.37 mg/l after administration of a curative dose of 20 mg/kg (cattle) and 5 mg/l after administration of a dose of 10 mg/l (pigs). The detected MIC₉₀ values for enrofloxacin and florfenicol were lower or similar to their C_{max} values, except for the majority of *E. coli* isolates. The MPC₉₀ values (the upper limit of MSW) exceeded it in all cases. For tulathromycin, relatively low serum drug concentrations is characteristic (Nowakowski et al. 2004). The C_{max} after administration of the recommended curative dose of 2.25 mg/kg was 0.5 mg/l for cattle and 0.6 mg/l for pigs and are below the MIC₉₀ value detected in this study, similarly to other reports (Godinho et al. 2005; Blondeau et al. 2012).

Based on the MIC and MPC of enrofloxacin, florfenicol, and tulathromycin, we identified the MSW of *P. multocida* and *E. coli* isolates from respiratory and enteric diseases of cattle and pigs and *S. aureus* isolates of dairy cattle mastitis to predict the likelihood of resistance selection. In this report, majority of isolates were susceptible or intermediately susceptible to all tested antimicrobials according to MIC determination, whereas the MPC values were usually high above breakpoints of susceptibility/resistance and the MSWs were detected as wide. Due to the fact that all tested bacteria may cause diseases of various animal species, including humans, these findings represent a major risk for the spread of resistant human and animal bacterial populations.

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