Detection of selected antibiotic resistance genes using multiplex PCR assay in mastitis pathogens in the Czech Republic

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Abstract

The aim of this research was to develop multiplex polymerase chain reaction assays for the detection of aminoglycoside (*strA*, *strB*), sulphonamide (*sulI*, *sulII*), tetracycline (*tetA*, *tetB, tetK, tetM, tetO*), macrolide and lincosamide (*msrA, ermA, ermB, ermC*, *mefA/E*) genes of resistance in mastitis pathogens (*Escherichia coli*, *Staphylococcus aureus*, *Streptococcus uberis*, *Streptococcus agalactiae* and *Streptococcus dysgalactiae).* Applying the established assays, we investigated the distribution of antibiotic resistance genes in the above mentioned species isolated from milk samples in the Czech Republic. Each assay consisted of seven pairs of primers. Six of them amplified fragments of antibiotic resistance genes and one pair a fragment of a species specific gene. Polymerase chain reaction conditions were optimized to amplify seven gene fragments simultaneously in one reaction. In total, 249 isolates were used, among which 111 were positive for *E. coli*, 52 for *S. aureus* and 86 for *Streptococcus* spp. The majority (60.2%) of bacteria carried at least one antibiotic resistance gene and 44.6% were multidrug-resistant. The designed multiplex polymerase chain reaction assays may be applied as diagnostic method to replace or complement standard techniques of antibiotic susceptibility testing in the mentioned pathogens.

S. uberis, *S. aureus*, *E. coli*, *S. agalactiae, S. dysgalactiae*

Bovine mastitis presents the biggest problem in milk farming (Cressier and Bissonnette 2011). Its worldwide spread endangers food safety, causing enormous economic losses for the dairy industry.

Over 150 different contagious and environmental microorganisms can cause mastitis (Kuang et al. 2009). The most common contagious pathogens are *Streptococcus agalactiae*, *Streptococcus dysgalactiae*, *Staphylococcus aureus*, and *Mycoplasma* spp. The most common environmental pathogens are *Escherichia coli*, *Klebsiella pneumoniae*, *Enterobacter aerogenes*, *Serratia* spp., *Proteus* spp., *Pseudomonas* spp., and other gram-negative bacteria, coagulase-negative staphylococci, environmental streptococci, yeast or fungi, *Prototheca* spp., *Arcanobacterium pyogenes*, and *Corynebacterium bovis* (Divers and Peek 2008).

Bzdil (2011) reports about the prevalence of mastitis agents in the Czech Republic. Data from 41 regions over a ten year period from 2000 to 2010 were evaluated, showing that the five most frequent pathogens were *Streptococcus uberis* (22.09%), *Staphylococcus aureus* (16.41%), *Escherichia coli* (7.01%), *Streptococcus agalactiae* (5.16%) and *Streptococcus dysgalactiae* (5.09%) (Bzdil 2011). In a similar study, which assessed mastitis pathogens in clinically healthy cows, results differ. There, the most prevalent pathogen was *Staphylococcus sciuri* (14.2%), followed by *Staphylococcus xylosus* (10.9%). *Staphylococcus aureus* was detected in 9.0% of the samples, *E. coli* in 6.6%. So called mastitis streptococci (*S. agalactiae*, *S. dysgalactiae*, and *S. uberis*) were discovered in 11.7% of all samples (Cervinkova et al. 2013).

The most common treatment strategy for mastitis is the use of antimicrobial drugs (Pyorala 2009); however, their wide use in veterinary medicine plays a significant role in

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the increasing resistance to them. As a consequence, the treatment of diseases caused by bacterial pathogens has become very difficult (Schwaiger et al. 2010). For this reason, determining the susceptibility of a microorganism to antibiotics is essential to proper treatment. Testing can demonstrate whether antimicrobial agents are useful against certain causative agents (Willey et al. 2008).

For *in vitro* testing of antimicrobial susceptibility, methods such as disk diffusion and broth dilution are commonly used (Kalmus et al. 2011). Despite the low cost and easy use, these methods have several disadvantages, such as labour and time consumption and investigator dependence (Driscoll et al. 2013; Hombach et al. 2013).

During the past few years several studies have reported about the application of polymerase chain reaction (PCR) based assays as a diagnostic tool for detecting mastitis-associated pathogens in milk (Shome et al. 2011; Shakuntala et al. 2012). The technique is highly sensitive, enables a high throughput and rapid results (Gao et al. 2011). At the same time, PCR is used to identify genes of resistance. Several multiplex PCR assays for detection of antibiotic resistant genes in *E. coli* (Sianglum et al. 2009), *S. aureus* (Strommenger et al. 2003) and streptococci (Malhotra-Kumar et al. 2005) have been developed. Since penicillins and cephalosporins are the most commonly used antibiotics for mastitis treatment (De Briyne et al. 2014), many PCR assays which detect beta-lactam antibiotic resistance genes have been designed (Pomba et al. 2006). There are further common genes of resistance. For *E. coli* they are: *tetA*, *tetB*, *strA*, *strB*, *sulI*, *sulII* (Srinivasan et al. 2006); for *S. aureus*: *ermA*, *ermB*, *ermC*, *msrA*, *tetM*, *tetK* (Gao et al. 2011; Parvizi et al. 2012); and for streptococci: *ermA*, *ermB*, *mefA/E*, *tetO*, *tetM* and *tetK* (Loch et al. 2005; Rato et al. 2013). These genes are responsible for bacteria not being susceptible to aminoglycoside (*strA*, *strB*), sulphonamide (*sulI*, *sulII*), tetracycline (*tetA*, *tetB, tetK, tetM, tetO*), macrolide and lincosamide (*msrA, ermA, ermB, ermC*, *mefA/E*) antibiotics. These are antibiotics widely used in the treatment of mastitis (Divers and Peek 2008).

Only few PCR assays were applied for the analysis of milk samples and exclusively for detecting genes of resistance in *S. aureus* (Gao et al. 2011; Parvizi et al. 2012).

The aim of this study was to develop multiplex PCR assays for detecting the most common aminoglycoside, sulphonamide, tetracycline, macrolide and lincosamide genes of resistance in bovine milk samples, originating from the pathogens *S. uberis*, *S. aureus*, *E. coli*, *S. agalactiae* and *S. dysgalactiae.* Additionally, the prevalence of these resistances in the Czech Republic was assessed.

Materials and Methods

A total of 218 cow milk samples were used in this study. The samples were obtained during milking. Desoxyribonucleic acid (DNA) extraction directly from milk samples and bacterial species identification were carried out by using "Thermo Scientific PathoProof™ Mastitis Complete-12 assay" (Thermo Fisher Scientific Inc. Waltham, MA USA).

Additionally, 31 bacterial samples isolated from milk with tested antimicrobial susceptibility were included in the assay. For this, colonies of 5 species of interest were obtained from the State Veterinary Institute in Olomouc. Seven of them were *E. coli*, six were *S. aureus*, six were *S. dysgalactiae*, five were *S. agalactiae,* and seven were *S. uberis*. Milk samples had been processed at the State Veterinary Institute in Olomouc with cultivation on blood agar (Trios s.r.o. Prague, Czech Republic) at 37 ± 1 °C for 42–48 h. The isolates were tested by the disk diffusion method. The antibiotic panel included streptomycin, tetracycline, neomycin, cefoperazon, novobiocin, clindamycin, cephalothin, amoxycilin/clavulanic acid, cotrimoxazol, oxacilin, colistin, ampicilin and cephalexin.

The DNA extraction from the obtained bacterial colonies was carried out using "DNeasy Blood & Tissue Kit (50) (QIAGEN®), which is suitable for such bacterial samples. The DNA concentration and purity were assessed using Nanodrop 2000 (Thermo Fisher Scientific Inc. Waltham, MA USA).

PCR was used for detection of aminoglycoside (*strA*, *strB*), sulphonamide (*sulI*, *sulII*), tetracycline (*tetA*, *tetB, tetK, tetM, tetO*), macrolide and lincosamide (*msrA, ermA, ermB, ermC*, *mefA/E*) resistance genes. To identify the pathogens, the following specific genes were chosen: *mrdB* for *E. coli*, *rRNA-16S* (ribosomal ribonucleic acid) for *S. agalactiae*, *rRNA-16S* for *S. dysgalactiae*, *Cpn60* for *S. uberis* and *rRNA-23S* for *S. aureus*. The primers

Table 1. List of primers.

Gene	Primer name	Sequence	Product size (bp)	Reference GenBank
ermA	ermA-F	TCAATGGTTGATGTCGTTCA	165	X03216
	ermA-R	AGAAGGGATTTGCGAAAAGA		
ermB	ermB-F	TTTTTGAAAGCCATGCGTCT	201	NC_012926
	ermB-R	CTGTGGTATGGCGGGTAAGT		
ermC	ermC-F	CAAAACGCTCATTGGCATTA	257	NC_024964
	ermC-R	ATCGTCAATTCCTGCATGTT		
strA	strA-F	CCGTCAATCCCGACTTCTTA	263	NC_001740
	strA-R	CCAGTTCTCTTCGGCGTTAG		
strB	strB-F	CGGTCGTGAGAACAATCTGA	313	NC_001740
	strB-R	ATGATGCAGATCGCCATGTA		
sulI	sulI-F	GACGAGATTGTGCGGTTCTT	350	X12869
	sulI-R	AGGGTTTCCGAGAAGGTGAT		
sulII	SulII-F	CCGTCTCGCTCGACAGTTAT	399	M36657
	sulII-R	ATTTGCGCGAAACAGACAG		
tetA	tetA-F	TGTCCGACAAGTTGCATGAT	178	X00006
	tetA-R	CCTTGAACGGCCTCAATTT		
tetB	tetB-F	CTCCTTGGCTTGGAAAAATG	229	NC_018998
	tetB-R	AACCAACCGAACCACTTCAC		
tetO	tetO-F	TAGCGGAACATTGCATTTGA	290	NC_012926
	tetO-R	TTTCTGTAAGTGCCCCAAGC		
tetM	tetM-F	AGGGCATCAAGCAACATTTC	366	NC_017331
	tetM-R	TCGAGGTCCGTCTGAACTTT		
tetK	tetK-F	CCCACCAGAAAACAAACCAA	439	NC_019148
	tetK-R	CCCTTCACTGATTATGGTGGT		
mefA/E	mefE-F	CGTATTGGGTGCTGTGATTG	248	U83667
	mefE-R	TATGCACAGGCGTTCCATTA		
msrA	msrA-F	AAGGCTTGTCCGCAATACAC	320	X52085
	msrA-R	CCATTACCCCCAATAAGTGC		
23S rRNA (aur23)	aur-F	CTAAGGTGAGCGAGCGAACT	110	X68425
	aur-R	CCTATTCACTGCGGCTCTTC		
cpn60	ube-F	ATCAGCCGCAGTTGAAGAAT	105	AF485804
	ube-R	TCCCCAACTTTTTCTGAACG		
rRNA-16S (dys16)	dys-F	AAGAATGATGGTGGGAGTGG	104	AB002488
	dys-R	CGCTCGGGACCTACGTATTA		
rRNA-16S (aga16)	aga-F	TTACCAGGTCTTGACATCCTTCT	116	DQ232512
	aga-R	GACTTAACCCAACATCTCACGAC		
mrdB	col-F	GTCCATCTCGATCCCACAAT	113	NC_000913
	col-R	CCGATTTTACGCTCCATCAT		

(Table 1) were designed using Primer3 software and checked for selfdimers, dimers and hairpin structures. The PCR mixture (total volume 10 µl) consisted of 5 µl of PPP Master Mix (Top-Bio, Prague, Czech Republic), 1.4 µl of primer mix (0.1 µl of each primer [10 pmol], see Table 2), 3.1 µl of PCR water and 0.5 µl of the template DNA (20 ng/µl).

PCR was performed under the following conditions: initial denaturation at 95 °C for 1 min, 35 cycles of denaturation at 95 °C for 30 s, primer annealing at 60 °C for 30 s and elongation at 72 °C for 30 s, and final elongation at 72 °C for 7 min. Reaction products were detected using a 2% agarose gel with ethidium bromide.

All PCR fragments had the correct size. The primers were validated using DNA from genotypically defined isolates which were obtained from the Veterinary Research Institute (Brno, Czech Republic). The isolates were also used as positive controls. The products of the PCRs were sequenced and data were compared to corresponding sequences in the GeneBank using the BLAST algorithm available at the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov).

Table 2. Multiplex polymerase chain reaction assays made according to targeted bacterial species.

Species	*Escherichia coli*	*Staphylococcus aureus*	*Streptococcus uberis*	*Streptococcus agalactiae*	*Streptococcus dysgalactiae*
name of gene	*sulII* (399)	*tetK* (439)	*tetK* (439)	*tetK* (439)	*tetK* (439)
(product	*sulI* (350)	*tetM* (366)	*tetM* (366)	*tetM* (366)	*tetM* (366)
size in bp)	*strB* (313)	*msrA* (320)	*tetO* (290)	*tetO* (290)	*tetO* (290)
	strA (263)	*ermC* (257)	*mefA/E* (248)	*mefA/E* (248)	*mefA/E* (248)
	tetB (229)	*ermB* (201)	*ermB* (201)	*ermB* (201)	*ermB* (201)
	tetA (178)	*ermA* (165)	*ermA* (165)	*ermA* (165)	*ermA* (165)
	mrdB (113)	aur23 (110)	*cpn60* (105)	aga16 (116)	dys16 (104)

Results

Strains with tested antimicrobial susceptibility

For the 31 isolates obtained from the State Veterinary Institute in Olomouc, results of the antimicrobial susceptibility test and PCR were compared. A good correlation was observed between phenotypical resistance and detected genes. However, four isolates with the detected genes were not resistant to the corresponding antibiotics. Three *E. coli* isolates, one with detected *strA* and *strB* genes, the second and the third with detected *sulII* gene, and one *S. aureus* isolate with detected *ermB* gene did not show resistance to corresponding antibiotics.

Four isolates of *E. coli* were resistant to tetracycline and carried the *tetA* gene. Four isolates were resistant to sulphonamide, but six carried the *sulII* gene and two additionally *sulI*. Five were resistant to streptomycin (aminoglycoside), but six carried both *strA* and *strB* genes.

Four isolates of *S. aureus* were resistant to tetracycline, all of them carried the *tetM* gene and one additionally *tetK*. One isolate was resistant to lincosamide and carried the *ermA* gene. At the same time, an isolate without resistance to lincosamide carried the *ermB* gene.

Sixteen isolates of streptococci were tetracycline-resistant. Nine of them carried the *tetM* gene and seven carried *tetO*. Eight isolates were resistant to lincosamide. Two carried the *mefA/E* gene and six carried *ermB*.

Strains without tested antimicrobial susceptibility

In total, 218 samples which contained species of interest were used. Following numbers of species were identified: 104 *E. coli,* 46 *S. aureus,* 25 *S. agalactiae,* 11 *S. dysgalactiae*, and 32 *S. uberis.*

The genes most often detected in *E. coli* were *strA* in 41 samples (39.4%) and *strB* in 52 samples (50.0%). This was followed by *tetB*, which was found in 31 (29.8%) samples. *SulI* and *sulII* were detected in 29 (27.9%) and 26 (25.0%) samples, respectively. T*etA* was

found least frequently – only in 22 (21.2%) samples. In total, 9 *E. coli* isolates carried five antibiotic resistance genes, 12 carried four genes, 7 carried three genes, 14 carried two genes and 11 carried one gene, in different combinations. In 8 samples all six antibiotic resistance genes were detected. No resistance genes were identified in 42 *E. coli* isolates.

For *S. aureus*, *msrA* was detected in 16 (34.8%) samples, *ermC* in 13 (28.3%), *tetM* in 12 (26.1%), *tetK* in 7 (15.2%), *ermB* in 4 (8.7%) and *ermA* in 2 (4.3%) samples. In total, only one *S. aureus* isolate carried five genes, 3 carried four genes, 8 carried three genes, 4 carried two genes and 5 carried one gene, in different combinations. No sample had all six genes. No sought genes were found in 25 samples.

Among streptococci, the most common genes were *tetM* and *ermB*, which were detected in 32 (47.1%) and 24 (35.3%) samples, respectively. *TetK* was found in 16 (23.5%) samples, *tetO* in 11 (16.2%), *mefA/E* in 9 (13.2%) and *ermA* in 6 (8.8%). In total, two isolates carried five genes of resistance, five carried four genes, 13 carried three genes, nine carried two genes and 11 carried one gene. No sample contained all six genes. In 28 isolates none of the sought genes was found.

Discussion

Despite the simplicity of the phenotypical susceptibility testing methods, the necessity to wait for the results for 48 h or more is a big disadvantage (Martineau et al. 2000). Multiplex PCR assay which simultaneously allows for the detection of several genes in a single reaction, has the advantage of identifying genotypic resistance to several antibiotics more rapidly and reliably (Choi et al. 2003). In this study, five multiplex PCR assays have been developed to test for the presence of *S. uberis*, *S. aureus*, *E. coli*, *S. agalactiae* and *S. dysgalactiae* (Table 2) and determine their antibiotic resistance by detecting the related resistance genes. The PCR assays allow highly accurate evaluation of antimicrobial resistance of mastitis pathogens and establishment of effective antibiotic therapy for cows with the disease. The assays could also be used for detection of the genes in samples of another origin.

Another aim of the study was to investigate the distribution of genetic resistance genes in the above mentioned species in milk samples in the Czech Republic. In our study, a total of 249 samples were analyzed. Among them 111 were *E. coli*, 52 *S. aureus* and 86 *Streptococcus* spp.

Of the *E. coli* isolates, 43 did not carry any gene of resistance. This contrasts with Srinivasan et al. (2007) who found at least one resistance gene in each investigated sample. On the other hand, their results show that the majority of *E. coli* (90.7%) was multidrug resistant. We also detected a multidrug resistance in the majority of samples, but only to 51.4%.

In a considerable number of *E. coli* isolates, *strA* (42.3%) and *strB* (52.3%) genes were amplified, which goes against the results of two other studies (Lanz et al. 2003; Srinivasan et al. 2007). *SulI* and *sulII* were detected in 27.9% and 28.8% of the isolates, respectively. Karczmarczyk et al. (2011) found similar results for the first gene, but the second one was found in 90% of isolates. Srinivasan et al. (2007) detected the mentioned genes only in 8% of all isolates. In their study, *TetA* (23.4%) and *tetB* (27.9%) were also detected at higher frequencies than in that of Srinivasan et al. (2007). Karczmarczyk et al. (2011) found *tetB* at a similar amount of *E. coli* as we did, but the presence of *tetA* was twice as high. Skockova et al. (2012) revealed the mentioned tetracycline resistant genes in highly varying amounts over a longer period.

Almost one third of *S. aureus* carried *tetM* (30.8%), whereas *tetK* was detected only in 15.4%. Similar results for tetracycline resistant genes have been shown in other reports (Gao et al. 2011, 2012). However, Kumar et al. (2010) observed a significantly higher prevalence of *tetK* compared to *tetM*.

Molecular analysis also revealed a high number of isolates with *msrA* (30.8%) and *ermC* (25.0%) genes, but quite few with *ermB* (9.6%) and *ermA* (5.8%). Gao et al. (2011, 2012) show in both their studies similar results concerning *ermC*, while in one study *ermB* was detected at a low rate, comparably to our results, and in another one *ermB* and *ermA* were not found at all. Kumar et al. (2010) revealed *msrA* in about a quarter of samples, while *ermA* and *ermC* could not be detected. In the work of Parvizi et al. (2012) *msrA* was found in 40% of the isolates and three other resistance genes in 20% each.

In 86 samples of *Streptococcus* spp., 57 (66.3%) carried at least one of the selected resistance genes. A study by Ruegg et al. (2015) demonstrated similar numbers, where 52.6% of samples were positive. Genes responsible for tetracycline resistance were detected in many samples. The gene *tetM* was present in almost half of the isolates - 41 (47.7%), *tetO* in 18 (20.9%) and *tetK* in 16 (18.6%). Similar results were shown for *tetM*, which was also the most frequent resistance gene discovered (Ruegg et al. 2015). This contrasts with the outcome of Rato et al. (2013), who report *tetK* as the most often detected tetracycline resistant gene. The most common macrolide resistance determinant was *ermB* (34.9%), followed by *mefA/E* (12.8%), and *ermA* (7.0%). The same ranking was shown in a report of Malhotra-Kumar et al. (2005). Combining the outcome of all these studies, it can be concluded that the distribution and relative frequency of genes of resistance in the same bacterial species are highly variable in different regions. Therefore, it is essential to test locally for every region for one cannot rely on data obtained from samples elsewhere.

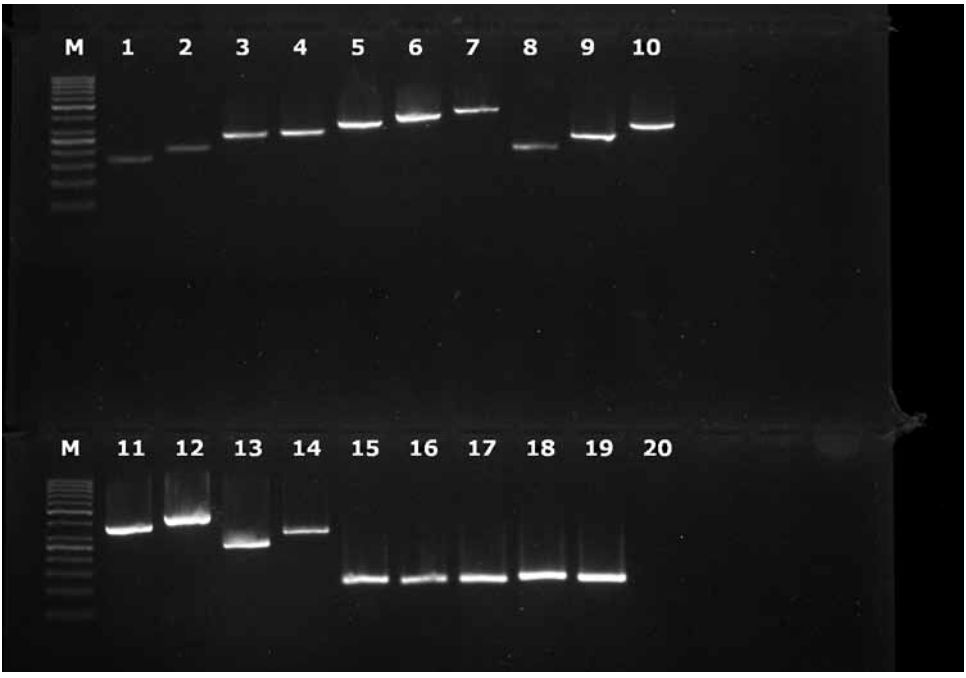

Fig. 1. Gel electrophoresis of multiplex polymerase chain reaction for detection of antibiotic resistance and pathogen specific genes in cow milk samples
Line: M – 50bp DNA (desoxyribonucleic acid) ladder; 1 – *ermA*, 2 – *ermB*, 3 – *ermC*, 4 – *strA*, 5 – *strB*, 6 – *sulI*, 7 – *sulII*, 8 – *tetA*, 9 – *tetB*, 10 – *tetO*, 11 – *tetM*, 12 – *tetK*, 13 – *mefA/E*, 14 – *msrA*, 15 – *rRNA-23S* (ribosomal ribonucleic acid) (aur), 16 – *cpn60* (ube), 17 – *rRNA-16S* (dys), 18 – *rRNA-16S* (aga), 19 – *mrdB* (col), 20 – negative control (water)

Another thing worth mentioning is the detection of genes of resistance in strains which did not show phenotypical resistance. This phenomenon could be explained by the fact that genes are not the only factors responsible for developing resistance to antibiotics (Cengiz et al. 2015).

The multiplex PCR assays developed in this study showed high sensitivity and specificity for *S. uberis*, *S. aureus*, *E. coli*, *S. agalactiae* and *S. dysgalactiae* (Fig. 1). The method is likely to be helpful for the rapid screening of antibiotic resistance. This research also reported the prevalence of aminoglycoside (*strA*, *strB*), sulphonamide (*sulI*, *sulII*), tetracycline (*tetA*, *tetB, tetK, tetM, tetO*), macrolide and lincosamide (*msrA, ermA, ermB, ermC*, *mefA/E*) resistance genes in the bacteria isolated from milk samples of dairy cows in the Czech Republic.

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