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A NEW REAL-TIME PCR ASSAY FOR RAPID IDENTIFICATION OF THE *S. AUREUS*/MRSA STRAINS

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Abstract

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The Methicillin-resistant *Staphylococcus aureus* (MRSA) with the livestock-associated MRSA (LA-MRSA) are of great interest to scientists and general public. The aim of our study was to present a new more rapid and reliable diagnostic method working on the RT-PCR platform applicable for monitoring of MRSA/*S. aureus*. The parallel testing of the *S. aureus* specific *nuc* gene sequence and the *mecA* gene sequence was utilised for this purpose. A collection of ten *S. aureus*/MRSA reference strains, fifteen genetically related non *S. aureus* reference strains and fifty-six environmental samples was employed for estimation of the assay performance and parameters. The environmental samples acquired in the Czech livestock farms were represented with the livestock and human nasal mucosae or skin swabs, the slaughter meat swabs and were chosen preferentially from individuals with previously confirmed or suspected positive MRSA/*S. aureus* cases. The classic selective cultivation approach with the biochemical test and agar disk diffusion test was accepted as reference diagnostic method. As there were no culture positive samples that were negative using RT-PCR, our method featured with 100% sensitivity in comparison to reference method. The limit of detection allowed to identify from tens to hundreds copies of *S. aureus*/MRSA genome. Further, the RT-PCR assay featured with 100% inclusivity and 95% exclusivity at Cq value below 30. These parameters suggested on powerful and reliable diagnostic method with real potential of practical utilisation. We consider our method as ideal for testing of individual suspected colonies, when the results can be acquired in less than 1.5 hour.

RT-PCR, MRSA, *S. aureus*, livestock

The *S. aureus* with MRSA represent pathogens with significant impact on food safety, human and animal health. At livestock, *S. aureus* belong to one of the most important mastitis pathogens. It colonises the skin and mucous membranes of living organisms and it is regularly detected also in the slaughterhouse meat and final food products. With regard to long-term livestock veterinary-service utilising antibiotic treatment inducing unfavourable selective pressure, the environment of farm animals and the milk and meat industry can be a possible source for further spreading of *S. aureus* including MRSA. The livestock-associated MRSA (LA-MRSA) ST398 has been widely identified in live animals and retail meat in Europe (van Duijkeren *et al*., 2007; Vanderhaeghen *et al*., 2010). According to new findings, the LA-MRSA ST-398 can be account also for a relevant part of MRSA isolated from human patients in hospitals or specialized practises connected to livestock production. This indicates LA-MRSA is significant cause for human MRSA infection (Köck *et al*., 2013). The pattern of MRSA transmission between animal and human is still in focus of broad scientific community. Appropriate screening methodology of MRSA detection represents crucial tool for effective MRSA management and for containment of MRSA

spreading. Traditional methods of MRSA detection are labour-intensive and include approximately 3- to 4-day turnaround time. Moreover, different commercially available media detecting MRSA show differing accuracy values (Luteijn *et al*., 2011). Therefore, there is marked interest in the development of alternative time and cost-saving, effective approaches to detect MRSA. The reliable and rapid microbial diagnostic in veterinary could also improve the appropriate medication and thus support the successful animal recovery and reduce the animal morbidity and mortality. Different diagnostic tests are available for the identification of MRSA so far. It is generally accepted, that the combination of PCR with cultural screening is the most reliable way for the detection of MRSA (Aydiner *et al*., 2012). However, rapid molecular identification of MRSA based on detection of one or several *S. aureus* specific genes or its sequences accompanied by the detection of *mecA* gene, encoding the protein PBP2a conferring methicillin resistance, has been accepted as a basis for a couple of published diagnostic methods or commercial kits. Further, the real-time PCR (RT-PCR) approach utilising fluorescent labelled hydrolysis probes has become a standard for sophisticated and reliable microbiological diagnostic in the last decade. Besides that, the parameters of individual tests can differ. As revealed also with our in silico analyse, some of the available methods can produce false positive results. In this study, we present the performance of our newly designed RT-PCR assay detecting the *S. aureus*/MRSA.

MATERIAL AND METHODS

Bacteria:

A collection consisting of ten *S. aureus*/MRSA reference strains, fifteen genetically related non *S. aureus* reference strains (*Staphylococcus epidermidis*, *S. hyicus, S. haemolyticus, S. chromogenes, S. xylosus, S. warneri, Streptococcus agalactiae, S. uberis, S. intermedius, Listeria monocytogenes*, *Escherichia coli*, *Enterococcus spp*.,) and fifty-six environmental samples were analysed to validate the performance of the method. The reference strains were obtained with the help of the Czech Collection of Microorganism. The environmental samples were acquired from the livestock farms in the Czech Republic during the years 2012–2013. The biological material for microbial diagnostics was represented with the swabs from animal and farm personnel nasal mucosa, skin and from slaughter meat. The individuals with previously confirmed or recorded positive MRSA/*S. aureus* cases or with suspected *S. aureus* infection were selected preferentially. As a reference MRSA diagnostic method, microbial identification of the *S. aureus* strains composed of selective cultivation on the Baird-Parker agar (Oxoid Ltd., UK), coagulase test, blood agar hemolysis test and biochemical test (Staphytest, Pliva Lachema, CR) were applied. The methicillin/oxacillin resistance was investigated using the agar disk diffusion method with regard to CLSI (Clinical and Laboratory Standards Institute, USA) standards. Classic PCR targeted the *mecA* gene was applied for confirming the MRSA strains among acquired suspected isolates (Poulsen *et al*., 2003).

DNA sample preparation:

Five to ten colonies from the 24 h blood culture of every reference strains was transferred into clean eppendorf tube. The bacteria were resuspended in buffer with 20 mM Tris/HCl; 2 mM EDTA; 1% TRITON X-100 (pH 8); supplemented with 20 mg/ml lysozyme and incubated for 45–60 min at 37 °C. The NucleoSpin Tissue kit (Macherey Nagel Inc., France) with the standard protocol was applied for subsequent DNA isolation. The concentrations and quality of the DNA isolates were measured on the Nanodrop 2000 instrument (Thermo Fisher Scientific Inc., DE, USA). The swab samples transported in Amies medium (Oxoid Ltd., UK) were employed for inoculation of the blood agar plates. The suspected 24-hour colonies were resuspended in 250–500 µl of sterile deionised water, heated at 100 °C for 10 min and centrifuged. The DNA for RT-PCR was gained from the supernatant. A second protocol demonstrating rapid RT-PCR analysis procedure was applied for direct testing of the acquired swabs. In pre-enrichment step, the swabs were inserted in suspension of Baird-Parker medium and cultivated for 8 hours at 37 °C. The Baird-Parker suspension was prepared via filtration of the Baird-Parker medium, when the agar substance was removed. After the enrichment, 1 ml of the suspension was inserted in the sterile 1.5 ml tube and centrifuged. The supernatant was removed, the pelleted cells were resuspended in 250–500 µl of sterile water. The final isolation of DNA was performed with short heating and centrifugation as described before.

Real-time PCR assay:

The RT-PCR assay working with the fluorescent-labelled hydrolysis probes was developed. To design the *S. aureus* specific assay, the sequence specificity of the *nuc* gene was utilized (GeneBank AM990992.1). PCR detection of the methicillin resistance was based on the *mecA* gene sequence identifying (GeneBank NC002952.2). Tens of *nuc* and *mecA* gene sequence sources were checked for the sequence homology and for identification of the known SNPs to exclude them from the primer design. The primers and probes were designed using the primer3 software (www:frodo.wi.mit.edu/primer3), the BLAST application (http://blast.ncbi.nlm.nih.gov) was applied to test the assay specificity in silico. The fluorescent dyes of the probes were chosen with regard to allow single-tube PCR genotyping. The dark BHQ-1 quencher was selected for the efficient quenching. The sequences of the oligonucleotides with the expected size of PCR products are shown in Tab. I. PCR was performed in 25 µl reaction mixture

I: *The primer and probe sequences*

gene		sequence (5´-3´)	PCR (bp)
nuc	forward primer	GACCTTTGTCAAACTCGACTTCA	118
	reverse primer	ACACCTGAAACAAAGCATCCTAA	
	probe, sense (FAM-BHQ1)	TTCGTAAATGCACTTGCTTCAGGACCA	
mecA	forward primer	GAATGCAGAAAGACCAAAGCA	124
	reverse primer	TTTGGAACGATGCCTATCTCA	
	probe, antisense (HEX-BHQ1)	ACCGAAACAATGTGGAATTGGCCA	

using a SsoFast™ Probes Supermix (BioRad Ltd., CA, USA), with 400 nM of the *nuc* and *mecA* primer set, 200 nM of the FAM/*nuc* probe, 250 nM of the HEX/*mecA* probe and 1 µl of sample DNA. The time and temperature conditions were as follows: 95 °C/3min; 35 cycles of 94 °C/15s; 60 °C/50s. The Bio-Rad CFX 96 instrument and software were employed for the RT-PCR purpose. Based on the measured fluorescence signals, a method of the second derivative maximum was utilised for determination of the cycle quantitative (Cq) numbers. The efficiency of amplification (E) was calculated from the slope of the standard curves according to Livak and Schmittgen, (2001). A ten-fold dilution of the standards was utilised. The limit of detection was defined as the lowest quantity of DNA template per sample that could be distinguished from negative samples with 95% confidence. To avoid any false positive signal caused by contamination, a sample without template was analysed as a negative control. The positive control represented with the reference MRSA DNA sample was included to every PCR set. In the RT-PCR format utilising nonspecific SYBR green dye, evaluation of the PCR melting curve with the melting temperature (Tm) was included in the analysis to check the specificity of the PCR products.

RESULTS AND DISCUSSION

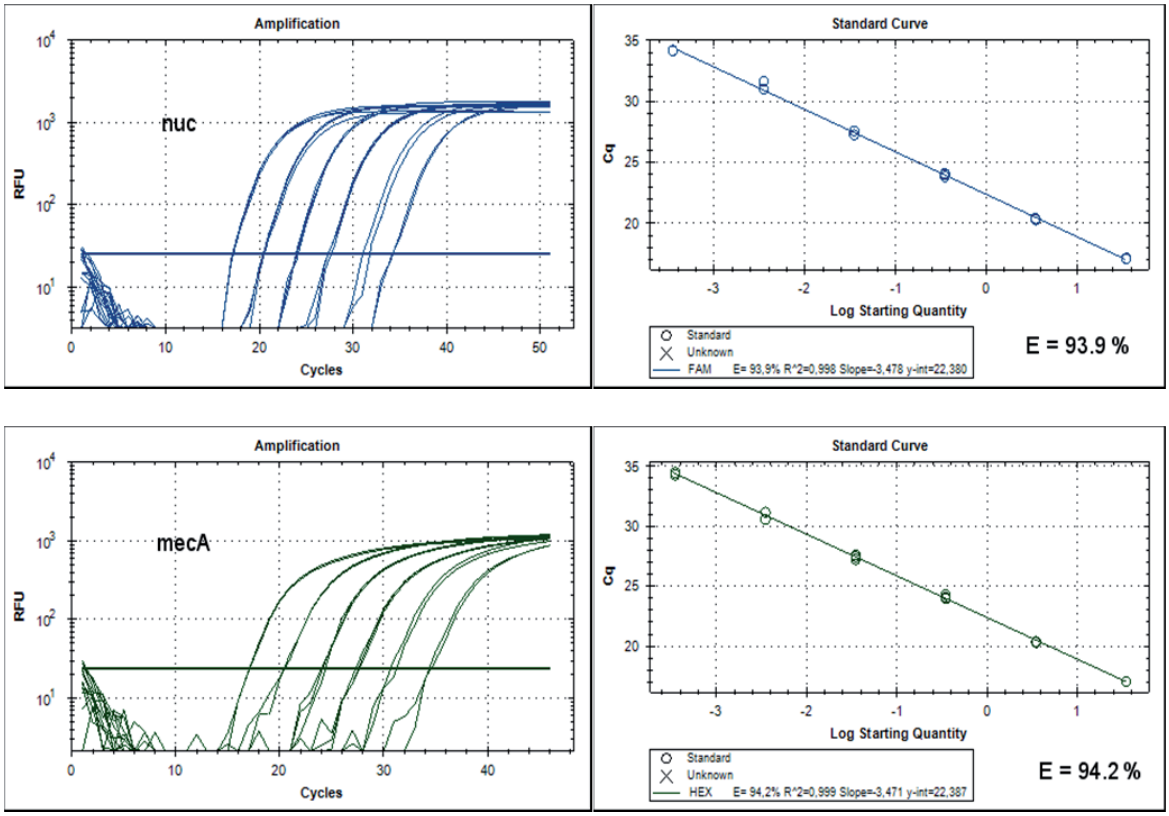
The new RT-PCR assay applicable for *S. aureus*/MRSA identification was developed. The method was constructed based on the *S. aureus* – characteristic *nuc* gene sequence. The additional single-tube monitoring of the *mecA* gene sequence enabled rapid identification of MRSA strains.

The primers were designed for 58–60 °C annealing temperature to save the universal thermal cycling parameters. This could be advantageous in case of multiplex RT-PCR or in case of parallel PCR testing of different targets. The design of the probes included the requirement for approximately 10 °C higher Tm (Melting temperature) than the Tm of the primers. This ensured that the complementary probes are fully hybridised during primer elongation. Here, the fluorescent reporters of the probes are released via 5' nuclease activity of the *Taq* DNA polymerase. The interruption of the FRET (fluorescence resonance energy transfer) between reporter and quencher causes increase of the fluorescence reporter signal. Finally, comparison of the post PCR fluorescence and the start-up basal fluorescence of the reporters in individual samples enables genotyping of analysed targets.

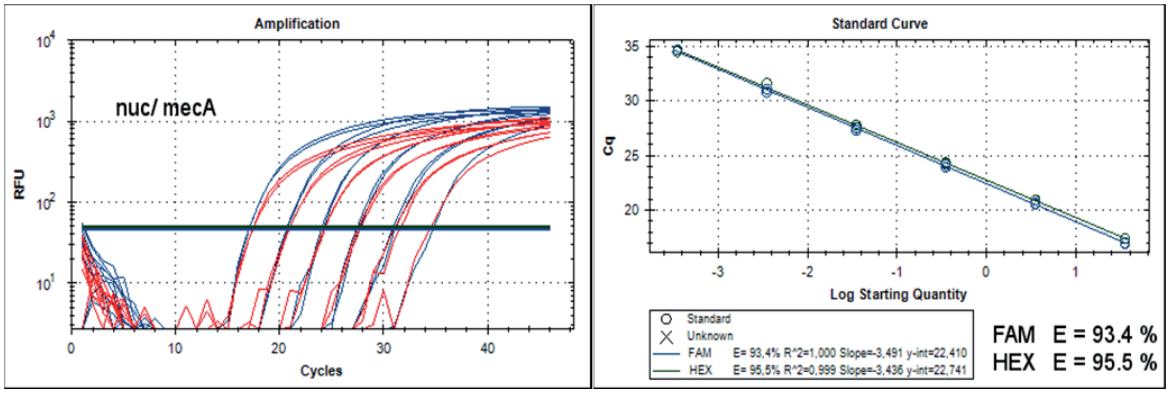
Experimental testing revealed, the both *nuc* and *mecA* PCR featured with full reproducible and robust amplification with sufficient E value (Fig. 1). As shown in Fig. 2, E values of the *nuc/mecA* primers estimated in individual *nuc/mecA* PCR runs and in duplex PCR showed equal values. This suggested on stabile duplex single-tube amplification without undesirable competitive interactions.

The RT-PCR diagnostic methods do not need post amplification manipulations and are accessible for automatic evaluation of the results. Using the allelic discrimination function (BioRad CFX 96 software) in our assay genotyping MRSA, the PCR results could be evaluated graphically as points on the FAM/ HEX probe axis (Fig. 3).

Testing of reference strains and suspected colonies originated from fifty-six environmental swab samples revealed, the both RT-PCR and reference culture method detected identical number of *S. aureus*/MRSA positive samples (In total thirty-eight *S. aureus* strains including nine MRSA strains). Therefore, our RT-PCR assay featured with 100% sensitivity as compared with the reference MRSA diagnostic method. Moreover, direct testing of enriched swab samples and primary selected suspected colonies provided identical results of *S. aureus* positive samples. The sensitivity of the method was further estimated using linearly diluted standard samples prepared from the reference MRSA strain. The obtained standard curves were linear over eight orders of magnitude. For the diagnostic purpose, the limit of the detection was estimated to be from tens to hundreds of *S. aureus* genome copies (calculated for *S. aureus* disposed of 2.9 Mb size genome; app. 3.5×10^{-7}–10^{-8} µg DNA per reaction). This is in agreement with findings of other authors (Alarcon *et al*., 2005; Ruimy *et al*., 2008). For reliable quantitative analysis, a hundreds of genome copies presented the minimum quantity of PCR template. In general, the RT-PCR sensitivity is considered to be higher as compared with the standard culture methods. Aydiner *et al*. (2012) found out nearly twice-time higher sensitivity of the commercial PCR based Detect-Ready MRSA assay (MDI, Kent, UK) in comparison to culture method. In their study, they did not utilise the enrichment step when testing the nose and throat swabs from patients.

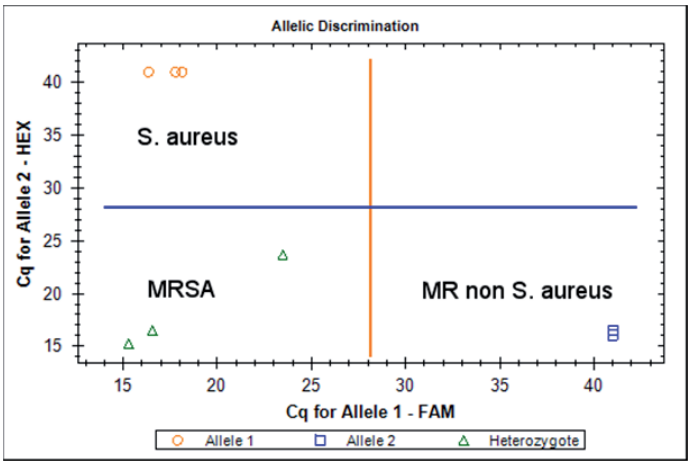


1: *Amplification efficiency (E) of the both nuc and mecA primers were estimated to be > 93 %. Testing of 10x diluted standard samples prepared from the MRSA reference strain, standards: 3.5 × 10¹–3.5 × 10⁻⁴ µg DNA. PCR (V=20 µl): nuc/mecA primers: 400 nM; FAM/nuc probe: 200 nM; HEX/mecA probe: 250 nM; 1x SsoFast™ Probes Supermix (BioRad, USA).*



2: *E value of the nuc/mecA primers estimated in duplex single-tube PCR run showed equal values as E in individual nuc and mecA PCR runs. Standard samples prepared from the MRSA reference strain: 3.5 × 10¹–3.5 × 10⁻⁴ µg DNA. PCR conditions were as in Fig. 1.*

However, the pre-enrichment step supplemented processing of swab samples in the rapid format of our assay contributed to additional elevating of the limit of the detection, as it ensured a higher yield of bacterial DNA. This enabled efficient testing of samples with minimal quantity of target pathogen. This type of samples can be expected also in case of slaughter meat swabs, animal and human skin swabs. In comparison to this, the samples like infected blood cultures feature with relatively high quantity of *S. aureus*, the enrichment step is therefore not necessary for the effective analysis (Ruimy *et al.*, 2008). We experimentally confirmed the short incubation at defined stable conditions should not change significantly the present pattern of bacteria. However, if preferred, the pre-enrichment step could be excluded to reduce the measurement error. In this case, the reduced sensitivity of the assay has to be accepted. If required CFU/ml units for the results interpretation, the transmission between known CFU/ml and acquired Cq levels or gene copy numbers has to be first estimated. Then, it is possible to predict the CFU/ml units in tested clinical samples. In agreement with this, Hein *et al.*,



3: *En example of automatic genotyping using the allelic discrimination function (BioRad CFX 96 software)*

(2001) estimated the correlation between log CFU and target gene copy numbers to range from 0.979 to 0.998, thus enabling calculation of the number of CFU by performing RT-PCR.

The evaluation of RT-PCR assay specificity in our study composed from the exclusivity and inclusivity test. All *S. aureus* and MRSA reference strains were identified correctly, what pointed out for 100% inclusivity of our assay. Further, all of the environmental samples tested positively as *S. aureus*/MRSA using the reference culture method has been tested positively also with the RT-PCR method. Analysis of the collection represented with genetically similar non *S. aureus* reference strains revealed the 90% exclusivity. Here, a small number (10 %) of non *S. aureus* strains possessing high genetic similarity to *S. aureus* generated positive PCR with high Cq values and could be false misidentified as samples of *S. aureus* with low template quantity near the detection limit of the assay. Definition of the Cq level included in analysis was therefore needful for proper evaluation of the results. To acquire maximally reliable results, we recommend acceptation of Cq values below thirty. In this interval, the assay exclusivity was almost absolute. Besides of mentioned custom adjustment improving the assay specificity, the basic specificity around 90% is full comparable with the performance of commercial accessible kits (Aydiner *et al.*, 2012).

Using an absolute quantification approach, the method allows performing of reliable quantitative *S. aureus* analysis so in individual as in swab mixture culture samples. The equal character of the standard curves of the both *nuc* and *mecA* assay indicated, the direct identification or quantification of MRSA in the swab samples is theoretically possible. In case of tested sample with identical results of Cq value for the *nuc* and *mecA* assay in our duplex RT-PCR, we can predict presence of MRSA in such swab sample. Unfortunately, the *mecA* gene could be present also in a number of coagulase- negative staphylococcal species. Therefore, the possible occurrence of methicillin resistant non *S. aureus* species has to be taken in account in this type of analysis. The problem arises in swab samples with different proportion of *S. aureus*, MRSA and MR-CNS (methicillin resistant coagulase-negative staphylococci), what can negatively affect the correct interpretation of the results. In this consequence, we considered the direct RT-PCR identification and quantification of MRSA in swab samples as not full reliable. However, it should be convenient method for primary screening of MRSA. The cultivated suspected *S. aureus*/ MRSA strains should be confirmed with repeated real-time PCR analysis on individual samples or with appropriate cultivation techniques. Quantification of the *mecA* gene in mixture samples could provide at least information about quantity of present methicillin resistant bacteria. In conclusion, we assume our RT-PCR approach detecting MRSA can find its main practical use at rapid testing of individual suspected colonies, obtained e.g. with the 24 hour cultivation on the blood agar.

To distinguish the MRSA and MRCNS, it is possible to design an additional PCR targeting the sequence at the junction of the *orfX* gene and staphylococcal chromosomal cassette mec (SCC*mec*) element, which is horizontally transferable among staphylococcal species (Ibrahem, 2010). This approach can take advantage of the fact that the SCC*mec* element carrying the *mecA* gene is integrated into the *S. aureus* genome at a unique site, at the end of *orfX*. There exist also commercial tests utilising this principle (Xpert MRSA assay, Cepheid, Sunnyvale, CA; BD GeneOhm MRSA assay, BD Diagnostics, Sparks, MD). Unfortunately, there exist several SCC*mec* types with different structure and composition (Chongtrakool *et al.*, 2006), what could affect the real possibility to design simple and reliable PCR test. Besides of that, the practical experience with the currently available dual labelled fluorescent probes and the RT-PCR instruments suggests, the single-tube RT-PCR analysis in case of four or more

primer and probe sets is rather complicated. The possible solution in form of additional individual PCR tests is not favourable, as it increases the labor intensity and the analysis costs. Existing commercial tests, detecting MRSA using single PCR targeting specific *orfX*-SCCmec sequence can be also a source of false positives. MSSA strains carrying at least one terminus of an SCC*mec* element, but lacking a *mecA* gene have been reported (Katayama *et al.*, 2005; Donnio *et al.*, 2007; Wong *et al.*, 2010; Arbefeville *et al.*, 2011). Only a limited number of studies evaluating the performance of PCR assays for the detection of MRSA have included techniques for detecting positive results due to MSSA empty-cassette variants. In the study of Arbefeville *et al.* (2011), who evaluated performance of the Xpert MRSA assay, the prevalence of MSSA isolates carrying remnants of the SCCmec cassette without *mecA* was below 10%. The other limit of methods targeting the *orfX*/SCC*mec* junction originates in existence of SCCmec-like elements with similar termini, which may also give rise to false positives (Wong *et al.*, 2010). Existence of the *orfX* homologs in CNS has been described as well (Aydiner *et al.*, 2012).

In a group of published methods of MRSA identification, based on PCR detection of the *S. aureus* specific genes supplemented with the *mecA* gene detection, the sequences of the *femA* (Ruimy *et al.*, 2008), *femB*, 16S rRNA, *tuf*, *nuc* (Alarcon *et al.*, 2008; Kilic and Basustaoglu, 2011; Zhang *et al.*, 2008) or *coa* (Ahmadi *et al.*, 2010) gene have been utilised. Surprisingly, our in silico analyse revealed a possibility for false positive results when using some of the published or commercially available primer and probe sets. For example, both the *femA* and *femB* gene based detecting methods possess risk for false positive results, because of 100% homologous *femA*, *femB* sequences revealed with the BLAST application in some of the strains closely related to *S. aureus* (e.g. GenBank: AF269697.1, CP000029.1 – *S. epidermidis*, AP006716.1, U23711.1 - *S. haemolyticus*, AP010958.1, FN649414.1 – *E.coli*). In addition, our SNPs inspection in available *femAB* gene sequences detected presence of high number of SNPs, which could be located also in the annealing sites of published primers. This could negatively affect the performance of relevant PCR test too. Therefore, we consider the *nuc* gene sequence for more reliable target enabling construction of diagnostic method with higher performance. For future improving of our method, we propose to employ the *femB* gene sequence as an internal amplification control. Using a *femB*-specific probe labelled with appropriate fluorescent dye, a triplex RT-PCR could be performed.

The rapidity of our assay depended on type of tested samples and on protocol chosen for DNA isolation. If the cultivated samples were at disposal, the full RT-PCR test for one sample took something more than one hour when using simple heating and centrifugation for the DNA isolation, respectively two and half hour when using NucleoSpin Tissue kit (Macherey Nagel Inc., France). With acceptation of the pre-enrichment step at swab samples, the total time of analysis reached the nine and half hour. It is apparent the RT-PCR approach could be very timesaving for analysing a large number of sample sets.

CONCLUSIONS

As shown by experimental validation of our new method, it can become a part of control tools applicable for more effective monitoring of *S. aureus*/MRSA occurrence in livestock farms or food products. We consider our method for reliable under conditions described in the work. It can find its practical utilisation also in veterinary or human medicine. Antibiotic treatment is often inadequate, reducing the chances of effective treatment or creating unnecessary selective pressure. The fast RT-PCR assay detecting MRSA could support rapid administration of the most appropriate antibiotic for infections caused by *S. aureus*.

SUMMARY

Livestock animals may be colonized with MRSA and the zoonotic transmission of such MRSA to humans via direct animal contact, environmental contaminations or meat are a matter of concern. To eliminate its further spreading, the efficient, rapid and easy to perform method has to be accessible. In our work, we present the new RT-PCR method applicable for practical monitoring of *S. aureus*/MRSA occurrence. The method working with the fluorescent labelled hydrolysis probes targets the *S. aureus* specific *nuc* gene sequence and the *mecA* gene sequence. The performance of our assay was validated with the help of experimental testing of ten *S. aureus*/MRSA reference strains, fifteen genetically related non *S. aureus* reference strains and fifty-six environmental samples. The environmental samples were represented with the livestock and human nasal mucosae or skin swabs and the slaughter meat swabs. These samples obtained in Czech livestock farms were chosen preferentially from individuals with previously confirmed or suspected positive MRSA/*S. aureus* cases. As a reference diagnostic method, the selective cultivation approach supplemented with the biochemical test and agar disk diffusion test were utilised. Our new RT-PCR assay featured with 100% sensitivity in comparison to reference method. It allowed successful identification of target pathogen with minimal quantity from tens to hundreds copies of genome. Further, the presented RT-PCR assay featured with 100% inclusivity and 95% exclusivity at Cq value below 30. This suggested on reliable method with sufficient specificity. We

suppose, our method can be helpful in rapid monitoring of the *S. aureus*/MRSA strains occurrence. It worked properly especially when working with the individual suspected colonies, where the results can be acquired in less than 1.5 hour. Further, our RT-PCR protocol offers a possibility for quantitative analysis of *S. aureus* in the swab mixture samples.

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