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REP-PCR-BASED TYPING AS A TOOL FOR TRACKING OF MRSA INFECTION ORIGIN

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Abstract

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A performance of rep-PCR fingerprinting method with the (GTG)5 primer was explored in order to evaluate its practical application for confirmation the source of human staphylococci infection. A laboratory worker daily working with the MRSA and *Staphylococcus aureus* strains has been tested positively for MRSA nosocomial infection. The collection of nine MRSA strains held in the laboratory has been typed with the rep-PCR method. Analysis of the fingerprints with the unweighted pair-group method using arithmetic averages (UPGMA) clustering method revealed presence of six strain clusters with similarity lower than 85%. The fingerprint of MRSA strain infecting the human differed significantly from remaining fingerprints. This provided clear evidence that MRSA strain infecting the personal did not come from the laboratory strains collection. Our experimental results showed rep-PCR with the (GTG)5 primer as an effective tool applicable for differentiation of individual *S. aureus* strains. However, the reproducibility and discrimination power of the method depended on strict observance of the optimal PCR time and temperature profile and PCR composition as well.

rep-PCR, fingerprinting, MRSA, infection

S. aureus represents a species with possible occurrence on skin, skin gland and mucous membranes of live animals including human. The environment of farm animals was identified to be an important source of pathogenic methicillin resistant *S. aureus* (MRSA) strains, with potential to contaminate the food chain and infect the human (de Lencastre, 2011). Besides the farm personnel and veterinarians, the laboratory workers handling collected bacterial pathogens could be a target of bacterial infection as well. Reliable identification and selection of the individual strains provides a very valuable knowledge about the genetic relationship of strains, about their dissemination and evolution. The possibility of identifying concrete bacterial strain is very important for effective protection of human and animals against dangerous bacteria including multi-resistant strains. A number of phenotypic and genotypic methods intended for *S. aureus* characterization have been described (Štěpán *et al*., 2004). However, only several methods are suitable for successful, time and cost-effective realising of strain-specific identification or strain selection. Some of those methods could be practically applied also for confirmation, exclusion or determination of the bacterial infection origin. Repetitive element sequence-based PCR (rep-PCR) represents an easy-to-perform technique utilising primers targeted to repetitive sequences dispersed in bacterial genomes (Versalovic *et al*., 1994). In our work, we sought to explore the performance of rep-PCR with regard to its practical implication for MRSA strain distinguishing and for molecular tracking of the infection origin on a case study.

MATERIAL AND METHODS

S. aureus/MRSA identification:

A collection of eight MRSA strains collected and archived in the laboratory, presenting objective risk of the lab personnel infection, was included in the study. The strains originated from different milk and meat farms in Czech Republic. The selective cultivation on the Baird-Parker agar (Oxoid Ltd., UK) and biochemical tests (Staphytest, Pliva

Lachema, CR) were used for bacterial identification of the *S. aureus* strains. The methicillin resistance was investigated using the agar disc diffusion method with regard to CLSI (Clinical and Laboratory Standards Institute, USA) standards. The MRSA strains were confirmed with the PCR targeted to *mecA* gene (Poulsen *et al.*, 2003). The standard safety procedures to prevent contact with bacteria were carried out. A biohazard box, gloves and protect shield were routinely used during the work in laboratory. One *S. aureus* strain isolated from the laboratorian nasal swab was confirmed for MRSA. To elucidate the source of laboratory worker infection, the rep-PCR analysis was employed.

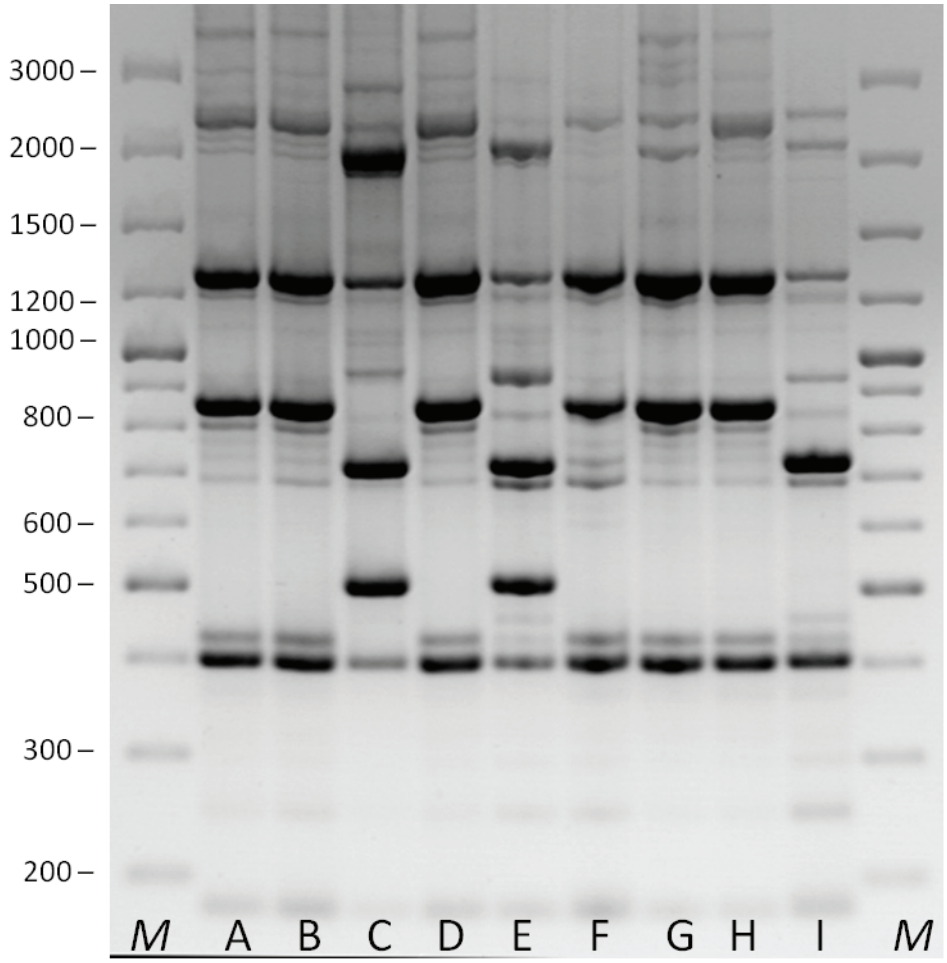
Rep-PCR analysis

The individual colonies cultivated on the Petri dishes were transferred into clean eppendorf tubes. As a preincubation step, the pelleted cells were resuspended in 20 mM Tris/HCl; 2 mM EDTA; 1% TRITON X-100 (pH 8); supplemented with 20 mg/ml lysozyme and incubated for 60 min at 37 °C. The NucleoSpin Tissue kit (Macherey Nagel Inc., France) was applied for DNA isolation and purification. The sample concentrations were measured on the Nanodrop 2000 instrument (Thermo Fisher Scientific Inc., DE, USA). To support the maximal reproducibility of the rep-PCR results, the identical quantity of template DNA (200 ng DNA) was added to each rep-PCR run. As an alternative, the alkaline extraction procedure was utilised for the genomic DNA isolation (bacterial cells were homogenised in lysis solution composed of 0.25% SDS; 0.05M NaOH and incubated at 95 °C for 15 min). The (GTG)5 primer (5´-GTGGTGGTGGTGGTG- 3´) was applied for the rep-PCR analysis. The PCR composition was as follows: 1 µm primer; 1.5 mM Mg^{2+}; 0.2 mM dNTP; 1x PCR buffer; 0.2 µl Taq DNA polymerase (Promega Ltd., WI, USA). The PCR time and temperature profile composed of following steps: initial denaturation 94 °C/7 min; 30 cycles of: 94 °C/1 min; 40 °C/1 min; 65 °C/8 min; final extension 65 °C/16 min. The rep-PCR products were separated electrophoretically on the 1.5% agarose gel (Fig. 1). The acquired data were normalised using the GelCompare II software (Applied Maths NV, Belgium), the unweighted pair-group method using arithmetic averages (UPGMA) clustering method with the Pearson and Jaccard correlation coefficient was utilized for the results interpretation and dendrogram construction (GelCompare II software, Applied Maths NV, Belgium).

RESULTS AND DISCUSSION

The obtained rep-PCR fragments ranged from 200 to 3800 bp (Fig. 1). The rep-PCR analysis revealed presence of six clusters of strains with similarity lower than 85 % (Fig. 2, Tab. I). The results of rep-PCR fingerprinting provided clear evidence, that the laboratory personnel was not infected with any of the MRSA strains collected in laboratory. The applied rep-PCR method was therefore effective tool applicable for tracking of infection origin.

Two independent DNA isolations and rep-PCR runs were performed simultaneously to validate the reproducibility of the method. The equal rep-PCR fingerprints including all strain-specific selective rep-PCR fragments were amplified successfully in independently repeated runs. To test the performance of the method, three different types of DNA polymerases were applied to get the results. The HotStar type DNA polymerase (Qiagen) reducing the rise of unspecific PCR products seemed not to be suitable for rep-PCR analysis and provided the worst results characteristic with poor rep-PCR amplification (data not shown). The remaining two protocols were represented with the PCR protocol working with classical Taq DNA polymerase of different manufacturers (Promega, Top-Bio). The individual PCR composition of these reaction sets was slightly modified. Here, we were able to distinguish significantly better results characteristic for classical protocol working with the unmodified Taq DNA polymerase. Moreover, the rep-PCR results pointed out on little different performance of the rep-PCR with regard to Taq DNA polymerase manufacturer. This indicated on a fact that individual experience and optimization of the rep-PCR could be helpful for its practical implementation. According to our results, the processing of native strain samples with the alkaline lysis followed with direct rep-PCR analysis without properly DNA isolation was sufficient for obtaining basic rep-PCR results. This is an optional advantageous alternative because of rapid sample processing and DNA isolation (Švec *et al.*, 2008). However, the heterogeneity in DNA template quality and concentration remained unknown and presented considerable problem for acquiring of reliable informative results. Here, we could observe creation of undesirable variability in generated fingerprints together with lower rep-PCR amplification efficiency. Applying of template DNA with different parameters in individual rep-PCR run complicated the correct interpretation of results in our study. This is in agreement with Švec *et al.*, (2010). Therefore, despite to very time-saving preparing of samples using described protocol, we considered this sample setup as not optimal for rep-PCR strain typing. In contrast to this, applying of purified DNA samples with equal quantity increased the reproducibility and the discrimination power of the rep-PCR. With regard to effort of achieving clear interpretable rep-PCR results, our experimental findings revealed little problematic evaluation of optimal rep-PCR product quantity which is loaded on the electrophoretic gel. In case of adding lower amount of rep-PCR product, some bands important for strains selection became undetectable. Therefore, we propose to apply higher amount of rep-PCR product for electrophoresis, although some rep-PCR bands could become overloaded. In case of testing higher number of

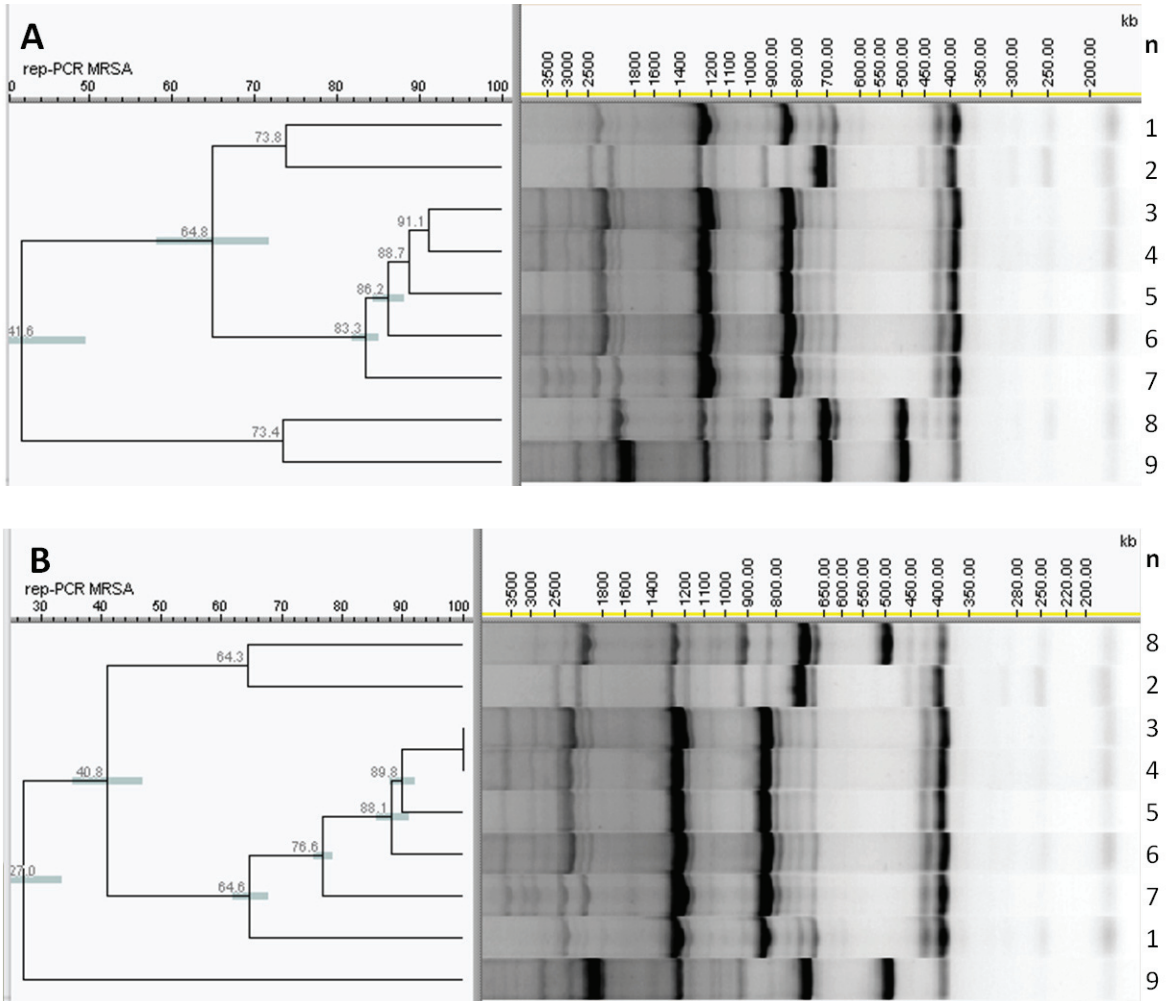


1: *Electrophoresis of MRSA rep-PCR products, raw data (line I – strain of human origin, M – ladder 100 plus (MBI Fermentas))*

strains, the method should be supplemented with appropriate analytical software. This allows acquiring of informative and reliable results. Despite to identical conditions during the whole rep-PCR analysis, little differences in band intensities of individual rep-PCR fingerprints has been observed. Visual inspection of the results together with its meaningful evaluation is therefore recommended. Applying of identical DNA isolation protocol, PCR chemistry and PCR instruments during all analyses could elevate the reproducibility of the method.

A detailed methodological evaluation of performance rep-PCR fingerprinting intended for *S. aureus* strain typing has not been investigated in the known literature. Of course, existing limits for rep-PCR typeability of individual bacterial strains could be expected. In fact, there is any 100% reliable molecular-genetic method usable for strain selection of the same species besides direct estimation of the nucleotide structure in tested genomes. Whole genome sequencing or partial genotyping of nucleic acids however still presents a high cost and high technically demanding method. In case of need for rapid, primary selection of tested strains for their further more detailed analysis, DNA sequencing of a large sample set is not acceptable approach. Here, the rep-PCR could be very effective, time and cost saving approach for primary analysis of tested samples. It could be a powerful diagnostic tool for individual strain typing and distinguishing without need for applying of additional analysis. As an analogous method for comparison genomic polymorphism between MRSA strains, the ribotyping on the RiboPrinter instrument could be employed. The method is considered as a high-throughput and sensitive tool for strain selection by many authors (Manga *et al*., 2011). In comparison to rep-PCR, the disadvantage of the method is very high requirement for equipment needs and high price of the analysis, which could not be comparable with low cost and simplicity of rep-PCR. Applying of frequently used pulsed field gel electrophoresis (PFGE) requires also higher demands on time, cost and laboratory equipment. Moreover, PFGE typing of MRSA strain belonging to clonal complex ST398 currently widespread in livestock seems to be unworkable (Stegger *et al*., 2011).

In our study, the rep-PCR analysis enabled distinguishing of six fingerprint clusters with homogeneity < 85%, while the strain of human origin



2: *Analysis of MRSA strains using the rep-PCR and the GelCompare II software (Applied Maths NV, Belgium): A – curve-based UPGMA clustering (Pearson correlation coefficient), B – band-based UPGMA clustering (Jaccard correlation coefficient), n – strain number, n (1, 3–9) – MRSA of animal origin, MRSA isolated from laboratory worker – strain n 2*

I: *Comparison of similarity (%) among tested MRSA strains, n – strain number, n (1, 3-9) – MRSA of animal origin, MRSA isolated from laboratory worker – strain n 2 (GelCompare II software, curve-based UPGMA clustering, Pearson correlation coefficient)*

n	1	2	3	4	5	6	7	8	9
1	100.00								
2	73.79	100.00							
3	68.94	53.17	100.00						
4	71.97	59.59	91.13	100.00					
5	76.10	64.60	88.32	89.06	100.00				
6	72.51	58.59	88.82	88.11	81.53	100.00			
7	70.02	52.19	86.13	82.91	85.98	78.20	100.00		
8	57.42	58.17	45.72	44.05	46.41	50.45	43.86	100.00	
9	30.19	32.55	39.29	31.34	32.47	37.88	32.62	73.39	100.00

differed significantly from remaining MRSA strains. However, the interpretation of the strains similarity differed little bit according to type of applied UPGMA analysis. The band-based cluster diagram with the Jaccard correlation coefficient provided results differing slightly from those constructed with the curve-based analysis and Pearson correlation coefficient. There is a possibility for applying of various correlation coefficients. Therefore, we propose to use the same type of statistical analysis to achieve a tool enabling comparison of a different sample sets. Applying of analysis with the Pearson correlation coefficient seems to be used most frequently in similar works of other authors.

The rep-PCR method has been successfully applied for typing and distinguishing of a number of different bacteria species inclusive Staphylococci species. Typing of *Staphylococcus* spp. to the strain level has been shown as realizable as well, a various platforms of rep-PCR fingerprinting methods have been utilised (Rantsiou *et al*., 2005; Iacumin *et al*., 2006). Moreover, Healy *et al*., (2005) introduced the rep-PCR-based automated system for bacteria typing – the DiversiLab™ system. This system has been tested and employed by several research teams for the experimental *S. aureus* and MRSA typing (Ross *et al*., 2005; te Witt *et al*., 2009; Grisold *et al*., 2010). The individual approach in the rep-PCR could be observed in the type of applied rep-PCR primer, which could be various. Koreňová *et al*., (2009) studied practical utilisation of rep-PCR with the (GTG)5 primer for identification of *Staphylococcus* spp. associated with sheep milk and meat. Švec *et al*., (2010), demonstrated high applicability of the rep-PCR working with (GTG)5 primer for identification of different Staphylococci species. Among the group of 41 species, all strains were typeable using the (GTG)5 primer. A few studies pointed out that rep-PCR primers could hybridise with additional similar sequences besides the specific repetitive target sequences. The reason of this could be the low annealing temperature of the applied rep-PCR protocols (Snelling *et al*., 1996; Wilson and Sharp, 2006). However, if the analysis is performed correctly at stable identical conditions, comprising from PCR time and temperature profile to composition of PCR reaction, this has no impact on quality and reproducibility of results interpretation.

CONCLUSION

The rep-PCR method working with the (GTG)5 primer could be an effective, fast and low cost analysis for *S. aureus* strain typing in case, if all requirements for precise laboratory work and all tools providing sufficient reproducibility of the analysis are carried out.

SUMMARY

S. aureus represents one of the most important pathogens markedly influencing the health status of human and domestic animals. As indicated worldwide epidemiological observation, the occurrence of dangerous, multi antibiotic resistant bacterial pathogens is on increasing level. Among the milk and meat livestock breeds tested positively for *S. aureus*, the methicillin resistant forms (MRSA) are regularly detected. To prevent their further dissemination, the existence of effective tools applicable for identifying and selection of individual strains is very important. In our study, we explored the performance of rep-PCR fingerprinting method with the (GTG)5 primer for typing of MRSA strains. Besides the practical evaluation of presented methods, we performed molecular tracking of MRSA infection origin on a case study. A nosocomial MRSA infection has been detected at laboratory personal, working with the collected *S. aureus* and MRSA strains of animal origin. The collection of all MRSA strains archived in laboratory comprising nine strains has been typed with the rep-PCR. The acquired data were normalised using the GelCompare II software (Applied Maths NV, Belgium). Statistical analysis based on the unweighted pair-group method using arithmetic averages (UPGMA) clustering method revealed presence of six strain clusters with similarity lower than 85%. The fingerprint of MRSA strain infecting the laboratory personal was unique and differed significantly from remaining fingerprints. Based on this knowledge, we obtained clear evidence that MRSA strain infecting the personal did not come from the laboratory strains collection. Our experimental results showed rep-PCR with the (GTG)5 primer as an effective, time and cost-saving approach, applicable for typing and selection of individual *S. aureus* strains. However, the strict observance of constant PCR time, temperature profile and PCR composition was absolutely important for acquiring of reproducible results with high discrimination power.

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