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Full Length Research Paper

Analysis of the polymorphism of *Staphylococcus* strains isolated from a hospital environment

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Nosocomial infections, despite the introduction of the numerous control systems, still constitute a considerable problem in hospitals. They are of great socio-economic importance due to the prolonged stay of patients in hospitals and high treatment costs. They also contribute to an increase in the death rate. For many years staphylococci have remained at the top of the list of microorganisms that cause infections in hospitals. The methicillin-resistant staphylococci (MRSA) strains that occur among both coagulase positive and coagulase negative (MRCNS) species are particularly dangerous. The aim of the presented paper was to determine the usefulness of the 16S rRNA gene sequence analysis and the region between the genes coding 16 S and 23 S rRNA for an examination of the diversity of *Staphylococcus* spp. isolated from a hospital environment and to perform a polymorphism analysis within and among the species. We also analyzed the polymorphism of 84 *Staphylococcus* strains and isolated from a hospital environment using PCR-RFLP and ITS PCR methods. A restriction enzyme analysis of the 16 S rRNA gene using *TaqI* enzyme did not show any considerable power of differentiation in relation to the *Staphylococci* strains examined. However, the variable region between genes 16 S and 23S rRNA showed a high degree of polymorphisms and allowed for the differentiation of the hospital strains examined. The results demonstrated the highest differentiation in *Staphylococcus aureus* strains. The examined strains originating from hospital environment are characterised by a high polymorphism.

Key words: ITS-PCR, *Staphylococcus* spp., hospital infections, 16 S rRNA, 23 S rRNA.

INTRODUCTION

Hospital infections, despite the introduction of numerous control systems, still constitute a considerable problem in hospitals in both developing and developed countries. They are of great socio-economic importance due to the prolonged stay of patients in hospitals and high treatment costs. They also contribute to and increase in the death rate (Jarvis, 1996; Ghanem et al., 2008; Cantlon et al., 2006). A hospital infection occurs when it has been contracted in a hospital and appears during the patient's stay in an institution, or within a specified period of time after the patient has been discharged. These infections may also afflict people who are not patients, i.e. medical staff, people visiting the ill or technical service workers of a hospital (Lis et al., 2009). Hospital infections are particularly dangerous for patients with the decreased immunity. The particular pathogencity of microorganisms present in hospitals which are exposed to numerous antibacterial agents and due to these conditions develop specific immunity mechanisms (Fiedotow and Denys, 2006). That is why it is critical to search for sources of infections in order to increase the efficiency of fighting them (Singh et al., 2006).

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The most commonly occurring multidrug resistant bacteria causing hospital infections are the methicillin-resistant strains of Gram-positive cocci from the *S. aureus* species (*methicillin resistant S. aureus* - MRSA). Other species from the *Staphylococcus* genus, coagulase negative and coagulase positive, both methicillin-resistant and methicillin-sensitive, are also common pathogens that cause hospital infections (El-Banna et al., 2010; Suka et al., 2008; Malikova et al., 2007).

Due to the pathogenic potential of these bacteria, effective methods for their identification *in vitro* are needed. At present diagnostic tests based on the evaluation of phenotypic features and a set of biochemical reactions and immunoreactions, as well as methods of analysing fatty acids are used (Sudagidan et al., 2005). Numerous problems associated with the credibility of these tests arise from the phenotype variability of the strains analysed. Additionally, they are mainly based on colorimetric techniques may lead to many mistakes. These methods enable the effective identification of *S. aureus* strains, but they often fail in cases of the coagulase negative strains. The research aimed at finding alternative identification methods that combine speed, reliability and low costs has been undertaken. The above criteria are met by the analysis of specific regions of genomic DNA which provide a lot more differentiating data. One of those alternative methods is the analysis of the 16 S rRNA gene sequence or ITS-PCR (Internally Transcribed Spacer PCR), where the region between genes coding 16 S and 23 S rRNA is amplified (Sudagian et al., 2005; Wojtyczka et al., 2010; Couto et al., 2001).

The sequence encoding bacterial 16 S rRNA is considered to be very conservative but in this region there are several variable regions. Changes in the individual nucleotides which may be characteristic for the given species, occur in them. As a result, it is possible to use this fragment (16 S rDNA) for a quick identification and determination of the bacteria's species affinity (Chakravorty et al., 2007).

For differentiation and identification of bacteria into different species, a variable fragment between the genes 16 and 23 S rRNA, where tRNA genes may appear, seems to be suitable. This fragment has various lengths not only between various species but also inside the genome of a given bacteria as copies of various alleles (Gurtler and Stanisich, 1996).

The objective of our research was to determine the usefulness of an analysis of 16 S rRNA gene sequence and the region between the genes coding 16 S and 23S rRNA for the evaluation of the diversity of *Staphylococcus* strains isolated from a hospital environment and analysis of polymorphism within the species and among the species.

MATERIALS AND METHODS

In the present research, 85 *Staphylococcus* strains were used. The strains were isolated from the air and surfaces of a hospital environment. Identification of all strains as species was made using biochemical tests API *Staph* (BioMerieux, France) (data not shown). Among the strains used for further analysis, 15 belonged to *S. epidermidis* species, 13 – *S. hominis*, 12 – *S. cohnii*, 11 – *S. haemolyticus*, 7 – *S. aureus*, 6 – *S. lugdunensis*, 5 – *S. warneri*, 4 *S. capitis*, 4 – *S. xylosus*, 2 – *S. chromogenes*, and 2 – *S. sciuri*. It was not possible to determine species affinity for three strains using biochemical tests. The *S. aureus* ATCC 25923 as a reference strain was used in the research. After isolating, the strains were kept for further research in a VIABANK system at -80°C.

The strains used in the research were cultivated on a blood agar plate (24 h at 37°C), then DNA isolation was done using a GenomicMini set (DNA-Gdańsk II s.c. Poland) with the addition of lysostaphin (400 u/ml). The concentration of isolated DNA was measured fluorometrically in a Qubit Fluorymetr (Invitrogen, Life Technologies). The obtained DNA was kept at -20°C for the further stages of the research.

Amplification was done using a HotStarTaqPlus MasterMix Kit (QIAGEN, Germany) in a reaction mixture with a final volume of 20 µl containing: 1 u polymerase of DNA HotStarTaqPlus, 1x PCR buffer with 15 mM $MgCl_2$, each 200 µM dNTP mixture, 1x Coral Load Concentrate, each 0.1 µM of suitable starters and ca. 200 ng isolated DNA. Two pairs of starters were used in the reaction. The first pair, 5'-GAAGTCGTAACAAGG-3' (G1) and 5'-CAAGGCATCCACCGT-3' (L1) for the ITS variable region, one starter was complementary until the end of the 5' sequence 16 S rDNA, and another until the end of the 3' sequence 23 S rDNA (3). The second pair, 5'-TACATGCAAGTCGAGCGAAC-3' (16Sf) and 5'-ACCTTCCGATACGGCTACCT-3' (16 Sr) for the 16 S rDNA fragment. PCR reactions were performed in a MJ Mini Personal Thermal Cycler (Bio-Rad, USA) using a program with the following parameters: initial denaturation at 95°C for 5 min, then 30 cycles as follows: denaturation at 95°C for 1 min, attaching of starters - for 16 Sf and 16 Sr pair at 65°C for 1 min and for the G1 and L1 pair at 54°C for 1 min, lengthening of starters at 72°C for 1 min. Amplification finished with a final lengthening at 72°C for 10 min.

Amplified DNA was separated in 3% agarose gel (Prona, Spain) for the region between 16 S rRNA and 23 rRNA, and in 1.5% agarose gel for the fragment of 16 S rRNA gene in a 1x TBE buffer. After the separation, DNA fragments were stained with ethidium bromide (0.5 µg/ml) and the obtained banding patterns were analyzed using the UVIDocMW digital imaging system (Uvtec, Cambridge, England).

The 954 bp PCR reaction product for the fragment of 16 S rRNA gene was analyzed using the *TaqI* restriction enzyme (Fermentas, Lithuania). For this purpose a reaction mixture containing 4 µl of the PCR reaction product, 3 µl of deionized water, 1 µl 10x of the *TaqI* buffer and 1 µl of the *TaqI* enzyme was prepared. Restriction analysis was performed in a MJ Mini Personal Thermal Cycer (Bio-Rad, USA) for 2 h at 65°C, and then for 20 min at 80°C. The products were exposed to electrophoresis in 1.5% agarose gel in a 1xTBE buffer with an insertion of ethidium bromide. Then, the obtained banding patterns were analyzed using the UVI-DocMW digital imaging system.

Statistical analysis of the obtained results of species identification was performed using the Shannon-Wiener Diversity Index and Simpson Diversity Index (http://www.changbioscience.com/genetics/shannon.html).

RESULTS

As a result of the performed amplification of 16 S rRNA genes, an amplimer of 954 bp was obtained. The amplification of the sole 16 S rRNA gene did not allow for differentiation of strains related to the differences in the

length of the examined region. All strains had an identical length of amplimers.

The restriction analysis using the *TaqI* enzyme was performed. After digestion of the 16 S rRNA gene amplification product, 5 different amplimers (data not shown) were obtained, and the scope of their fragment lenght after the analysis in the UVI DocMW programs was between 84 and 954 bp.

As a result of this analysis, we divided the isolated strains in relation to the similarity of their electrophoretic profiles. The main difference appeared with the biggest band. For some strains it was the band of 954 bp, and for others of 783 bp. There were also strains with bands of not only 783 bp, but also 954 bp.

In the next stage of the research, the obtained strains were analysed in relation to differences in the region between the 16 and 23 S rRNA genes. The electrophoretic profile for the ITS fragment demonstrated a more considerable diversity (Figure 1). The differences concerned both the length and the number of the emerged amplimers. Depending on the strain, the profile consisted of 1 to 11 main bands. The size of obtained fragments after the analysis in the UVI DocMW program was from 278 to 792 bp. The majority of strains demonstrated a diverse banding pattern with numerous ITS amplimer bands of various lengths; however strains with identical banding pattern were also observed. None of the examined strains showed a similar banding pattern to the *S. aureus* ATTC 25923 type strain.

The diversity of strains within individual species identified in the biochemical tests (data not shown) were also examined. Strains belonging to *Staphylococcus cohnii* and *Staphylococcus epidermidis* species were the least diverse. Their banding patterns were very similar for the majority of the examined strains in a given species. But even within these species, strains with a different electrophoretic profile could be found. The most considerable diversification of banding patterns within a species was obtained for the *S. aureus* species. It was not possible to find a dominating banding pattern which would be characteristic for this species. However, strains belonging to the species: *Staphylococcus hominis* and *Staphylococcus haemolyticus* demonstrated a moderate differentiation.

Statistical analysis using the Shannon-Weiner Diversity Index H = 2.215717 and Simpson Diversity Index D = 0.12221453 (Table 1) showed a high biological differentiation of the isolated strains.

DISCUSSION

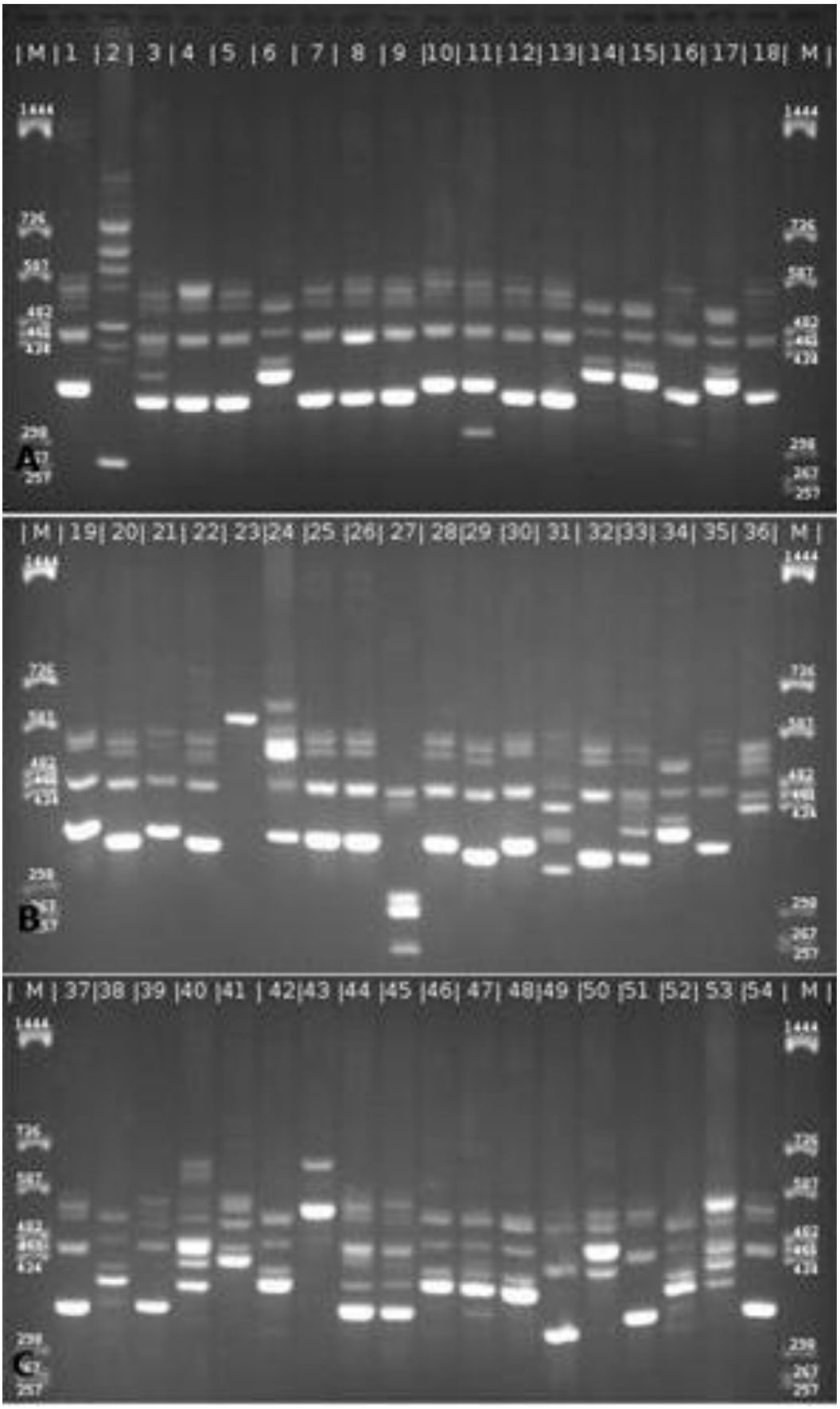
It has been widely described that the most useful method for the microorganisms differentiating is ribotyping, that is, analysis of different regions of the *rrn* operon, which is present in all bacterial organisms. The most commonly analysed fragments are: the region containing the 16 S rRNA coding genes and the region between the 16 S and 23 S rRNA coding genes (Wojtyczka et al., 2010; Gurtler and Stanisich, 1996; Krawczyk, 2007). The analysis of the 16 S rRNA gene is considered to be a standard identification method allowing for the taxonomical classification of the examined bacteria to family, genus and even species (Gurtler and Stanisich, 1996).

The results of our research concerning the banding patterns for the 16 S rDNA fragment did not allow for a differentiation of strains, and that is why this fragment was cut with the *TaqI* restrictive enzyme. Despite the restrictive analysis, the results did not allow the strains. In this case the PCR-RFLP method did not show any considerable differentiating power of the examined *Staphylococci'* strains, which confirmed the conclusions of Heikens et al. (2005) who claimed that genotypic identification based on the 16 S rRNA fragment has limited differentiating power because of the close genetic relation between the individual species within *Staphylococcus* genus (Heikens et al., 2005). Possibly the use of other enzymes or cutting this fragment with several restrictive enzymes simultaneously or doing a sequence analysis of chosen fragments might provide more considerable differentiating opportunities.

Genes coding ribosomal RNA may lose differentiating power in the case of lower taxonomical levels; however, the ITS variable region situated between the 16 S and 23 S rRNA genes may enable identification of microorganisms at the level of species or subspecies (Mora et al., 2003). In the next stage of the research, the obtained strains were analysed in relation to differences in the area between 16 and 23 S rRNA (ITS) using the ITS-PCR technique.

The electrophoretic profile for the ITS fragment demonstrated considerably more diversity (size and number of bands) than in the case of the 16 S rDNA section. Additional digestion of restrictive enzymes, suggested by some researchers (Mendoza et al., 1998), was not necessary. The majority of strains demonstrated an abundant banding pattern with numerous ITS amplimer bands of various lengths.

Couto et al. (2001) using the ITS-PCR method identified, through a comparison of the obtained banding patterns with the type strains pattern, 95.95% out of the 617 examined *Staphylococcus* strains originating in various hospitals. Strains were classified as 11 species. All of the examined species had a unique banding pattern; however, some of them demonstrated some similarities, which allowed for their division into 3 groups. The majority of the species were easy to identify through a direct comparison with type species profiles, and due to this fact, digestion with restrictive enzymes was not necessary. The observations of Couto et al. (2001) confirm our results i.e. by using the ITS-PCR method one may obtain a diverse electrophoretic profile for the ITS variable region, which allows the strains belonging to various species to be differentiated without the need for


| M | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |10|11|12|13|14|15|16|17|18| M |
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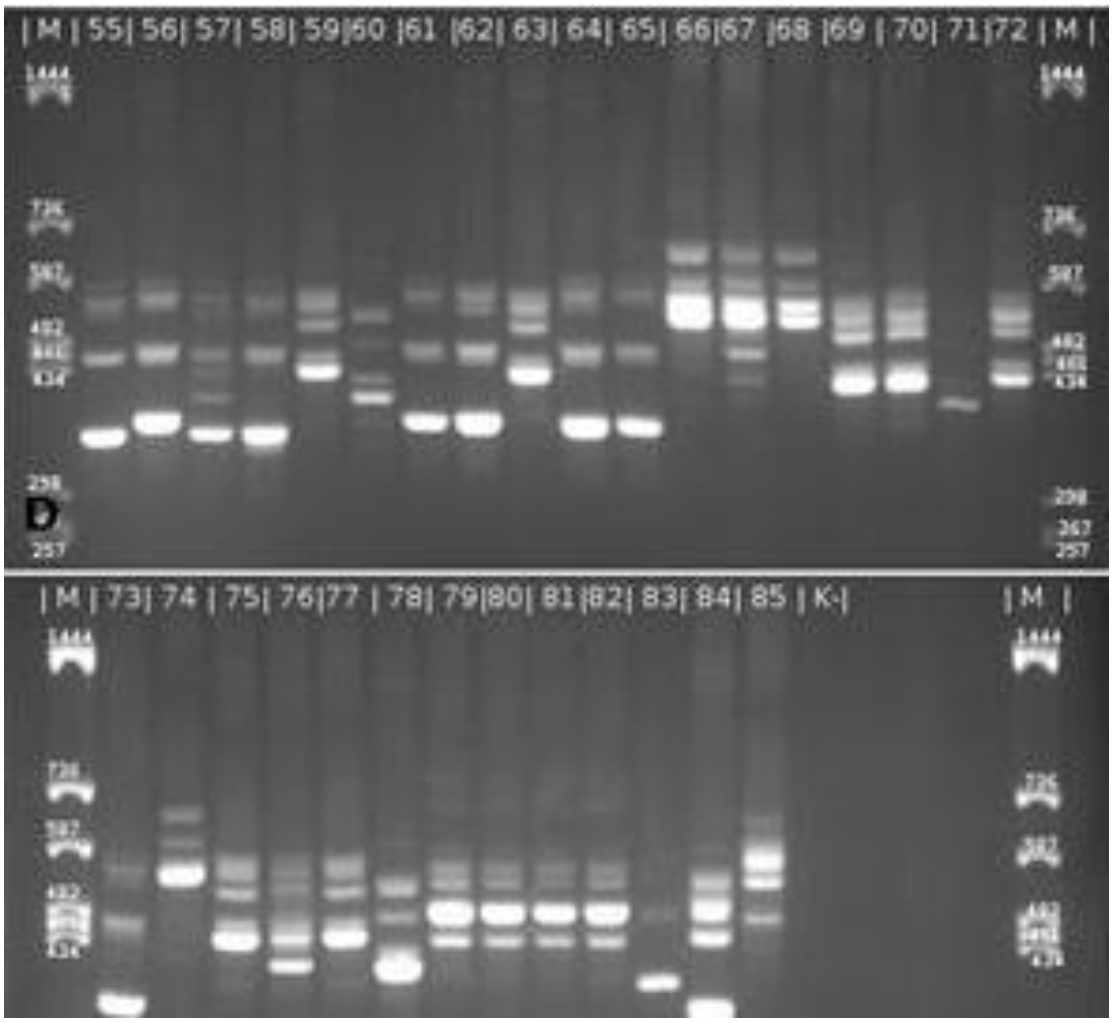

Figure 1. The electrophoretic profile for the ITS variable fragment. A- strains 1-18, B- strains 19-36, C- strains 37-45, D- strains 55-72, E-85; M- size marker, K(-) - negative control, *S. epidermidis* lanes - 8, 9, 12, 13, 20, 22, 29, 32, 37, 39, 42, 44, 51, 52, 54; *S. hominis* lanes -2, 3, 6, 11, 14, 15, 34, 61, 67, 73, 76, 78, 84; *S. cohnii* lanes – 1, 10, 16, 18, 21, 24, 25, 28, 30, 35, 64, 65; *S. haemolyticus* – lanes 33, 41, 43, 48, 59, 63, 66, 69, 72, 75, 77; *S. aureus* lanes – 40, 53, 58, 62, 68, 70, 74; *S. lugdunensis* lanes -17, 45, 55, 57, 60, 71; *S. warneri* lanes – 50, 79, 80, 81, 82, *S. capitis* lanes – 4, 5, 23, 31; *S. xylosus* lanes - 7, 19, 26, 83; *S. chromogenes* lanes – 46, 47; and *S. sciuri* – lanes 27,38; *S. aureus/haemolyticus* lanes – 36, 49; *S. aureus/S. xylosus* lane – 7; *S. aureus* ATCC 25923 lane 85.

digesting with restrictive enzymes.

Our results are similar in agreement with those presented by Mendoza et al. (1998), who demonstrated that amplification of the ITS variable region using starters for the gene fragment between 16 S and 23 S rRNA is suitable for the identification of 220 *Staphylococci* belonging to 31 species. The banding patterns of a diversified number (1-8) and quantity of bands (104-774 bp) were obtained. No differentiation inside the species containing a majority of the examined species was demonstrated, apart from *S. aureus,* which showed the highest polymorphism of the ITS region among all analysis staphylococci. In our research, the most considerable diversification of banding patterns within a species was obtained for strains from the *S. aureus* species. It was not possible to find strains with the same electrophoretic profile. Differentiation within the *S. haemolyticus* and *S. hominis* species was smaller, while *S. epidermidis* and *S. cohnii* turned out to be the least differentiated species. However, it is possible to divide the examined strains into groups in relation to the similarity of the banding pattern and a determination of the dominating electrophoretic profile in a given species, which could be useful in epidemiological research.

Dubey et al. (2009) in research on strains belonging to *S. aureus* species also noticed difference in the quantity and size of bands within this species after ITS fragment amplification. They also noticed similarities in the banding

Table 1. An analysis of the differentiation of *Staphylococcus spp*. strains using ITS PCR technique. Total number of strains =85.

Staphylococcus spp.	**Number of strains**	**Pi**	**ln(Pi)**	**Pi ln(Pi)**
Staphylococcus epidermidis	15	0.1786	-1.72277	-0.30764
Staphylococcus hominis	13	0.1548	-1.86587	-0.28877
Staphylococcus cohnii	12	0.1429	-1.94591	-0.27799
Staphylococcus haemolyticus	12	0.1429	-1.94591	-0.27799
Staphylococcus aureus	10	0.1071	-2.23359	-0.23931
Staphylococcus lugdunensis	6	0.0714	-2.63906	-0.1885
Staphylococcus warneri	5	0.0595	-2.82138	-0.16794
Staphylococcus capitis	4	0.0476	-3.04452	-0.14498
Staphylococcus xylosus	4	0.0476	-3.04452	-0.14498
Staphylococcus chromogenes	2	0.0238	-3.73767	-0.08899
Staphylococcus sciuri	2	0.0238	-3.73767	-0.08899
Shannon-Wiener Diversity Index		2.215717		
Simpson Diversity Index	D:	0.12221453		
	1-D:	0.87778544		
	1/D:	8.182333		
Evenness		0.92402583		

patterns of some strains. Diversity of banding patterns could be the evidence of the high polymorphism of bacteria belonging to the *S. aureus* species.

However, Villard et al. (2000) verified the usefulness of the variable region situated between the 16 S and 23 S rRNA genes for the identification of *S. xylosus* strains. In this research, all the species of the *Staphylococcus* genus exhibited a characteristic electrophoretic profile after amplification of the variable region (ITS), which enabled the differentiation between these species. All of the examined strains of *S. xylosus* had a unique banding pattern, which is easy to analyse and enables fast screening of unknown isolates.

Amplification of the ITS region showed how great variability is characteristic of bacteria belonging to *Staphylococcus* genus that exist in a hospital environment. However, it is possible to observe similarities in banding patterns, appearing as a result of electrophoretic division of the amplified ITS fragment, between some of the strains belonging to the same species. Sometimes, they form banding patterns which may be defined as characteristic for a given species. Despite the noticeable similarity some dissimilarities also exist. These may concern individual bands and be slight. In our research a strain with a species affinity not defined in biochemical tests and belonging to the *S. aureus* or *S. xylosus* species demonstrated some homology in the banding pattern with strains belonging to the *S. aureus* species, which might suggest that it belongs to this species and the biochemical analysis was probably wrong. A similar case is with one of the strains which belong to the *S. aureus* or *S. haemolyticus* species according to the biochemical tests. It demonstrates a more considerable homology with the banding patterns of strains belonging to the *S. haemolyticus* species. Another of these strains had a completely different electrophoretic profile from the rest of the strains belonging to the *S. haemolyticus* species. This may be evidence that it is a strain which shows similar biochemical properties to strains belonging to the *Staphylococcus haemolyticus* species, but in reality it does not belong to this species.

The obtained similarities in the banding patterns could show that the examined strains are phylogenetically related. Strains which exhibit completely different banding pattern from the one which is exhibited by the majority of a given species also exist. This may be an indication of the inaccuracy of biochemical tests which classified a given strain as a specific species. The dissimilarity of banding patterns of individual strains may also be caused by the high variability of hospital strains.

To sum up, it may be concluded that strains belonging to *Staphylococcus* genus, which come from a hospital environment are characterized by a high polymorphism (Wojtyczka et al., 2010). This may be analysed through amplification of the variable fragment situated between the 16 and 23S rRNA (ITS) coding genes.

Conclusions

1. The RFLP analysis of the ITS ribosomal operon fragment provides more opportunities for the differentiation of strains within the *Staphylococcus* genus than an analysis of the 16S rDNA fragment using the RFLP

method with the *Taq1* restriction enzyme.
2. In the examined hospital environment *S. aureus* was the most diverse species, whereas *S. epidermidis* and *S. cohnii* were found to be the least diverse species.
3. The ITS region may be successfully used not only for differentiation between the *Staphylococcus* strains, but also for differentiation between individual strains.

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