3. Abstract

Molecular epidemiology is the study of infectious diseases in relation to selected genetic characteristics of the microorganism causing the host infection. Molecular epidemiological studies help to understand the mechanisms of interaction between the host and the pathogen and define virulence factors, phylogenetic relatedness, and pathogenic microbial transmission pathways. The rods belonging to the *Enterobacteriaceae* family pose a significant threat among gram-negative bacteria, including *Escherichia coli* and multi-drug resistant strains of *Pseudomonas aeruginosa*. Among the methods of typing microbial microorganisms used in diagnostics, one can distinguish methods based on the analysis of phenotypic characteristics and genetic material of microorganisms. Genotyping methods have been used in microbiological diagnostics and epidemiological research - in detecting, identifying, and differentiating microorganisms. Replacement phenotypic methods with molecular techniques based on the polymerase chain reaction (PCR) for characterizing clinical isolates allowed to shorten the analysis time and reduce their costs and labor consumption. It also allowed for studying microorganisms whose laboratory cultivation is difficult or impossible.

The main aim of this dissertation was to improve the methodology of genetic differentiation of pathogenic strains of *Escherichia coli* and *Pseudomonas aeruginosa* using the TRS-PCR technique. This work is a continuation of the research on the TRS-PCR molecular method developed in the Laboratory of Molecular Genetics, used for the differentiation of clinical strains, based on the analysis of the similarity of band profiles. The interpretation of TRS-PCR band profiles was improved by introducing a numerical system in which the same profile was assigned a numerical value. This approach allows for a more accessible analysis of molecular profiles obtained for the tested strains and the possibility of comparing the results obtained in different laboratories. In order to determine the differentiation power of the new TRS-PCR methodology, the results were compared with those obtained from the MLST (for *E. coli* collections) and MLVA (for *P. aeruginosa* strains) analyses.

First, studies were conducted that allowed for developing and evaluating the usefulness of the TRS-PCR test using the $N_6(CAC)_4$ primer to differentiate uropathogenic *E. coli* strains. Considering the high reproducibility of this method, identical to that for the GTG-PCR test but lower than for the CGG-PCR, the CAC-PCR method allowed the grouping of 124 tested UPEC strains into 52 unique clusters.

The averaged analysis of the band profiles based on three TRS-PCR tests for UPEC isolates allowed for assessing their differentiating potential. Given the high value of the repeatability coefficient of 95%, 111 unique strains were identified from among 124 tested UPEC isolates. Such a result indicates a high efficiency of the differentiation of UPEC strains using averaged analysis based on the above-mentioned TRS-PCR tests.

TRS-PCR band profile analysis methodology was improved by introducing numerical classification for identical profiles obtained in individual TRS-PCR tests to differentiate the collection of uropathogenic clinical *E. coli* strains. The profiles with similarities above the calculated repeatability value were considered identical. As a result of comparing the strength of differentiation of the CAC-PCR method with the molecular tests carried out previously in the Laboratory for the studied collection of UPEC strains, based on the CGG- and GTG-PCR methods, 52 CAC-PCR classes, 86 GTG-PCR classes and 99 CGG classes were obtained. Subsequently, each *E. coli* isolate with a unique TRS-PCR class profile (CAC-, GTG- and CGG-PCR) was assigned to a separate TRS-PCR group. This approach to the interpretation of profiling results allowed for the differentiation of 121 unique isolates, differing in at least one TRS class, from among 124 uropathogenic *E. coli* strains available in the collection of the IBM PAN Molecular Genetics Laboratory. Differentiating clinical UPEC strains based on the numerical system of assigning TRS-PCR classes showed a better ability to differentiate isolates than the classical, averaged analysis of the band profiles.

In the next part of this work, a comparative analysis was carried out for several selected UPEC and STEC strains, using two different molecular typing techniques: based on the numerical classification TRS-PCR system and the MLST method on the scheme proposed by Wirth *et al*. The TRS-PCR analysis provided the results differentiating selected strains of *E. coli* like those obtained in the MLST analysis. However, the results suggest that the TRS-PCR method also allows for the differentiation of some strains with identical ST sequence profiles.

In this study, the usefulness of the methodology of comparing CGG- and GTC-PCR band profiles for determining the genetic diversity of clinical collections of *P. aeruginosa* strains isolated from patients with cystic fibrosis was tested for the first time. The determined values of the repeatability coefficients of the analyzes performed for both the CGG- and GTC-PCR tests were at a very high and similar level (93.3% and 95.3%, respectively), which means that the TRS-PCR analyses for the collection of *P. aeruginosa* may have a high differentiating potential. Of the 63 *P. aeruginosa* isolates tested,

27 unique strains were classified by CGG-PCR differentiation, while GTC-PCR differentiation allowed the identification of only ten unique isolates. This result allowed us to conclude that the CGG-PCR test has a better differentiation potential than the GTC-PCR method. Then, an averaged analysis of the similarity of the obtained band profiles for both TRS-PCR tests was performed, assigning 63 tested *P. aeruginosa* strains to 26 clusters.

Subsequently, the strains were assigned CGG- and GTC-PCR class numbers. *P. aeruginosa* strains with band profiles displaying similarity above the repeatability value for both CGG- and GTC-PCR tests were treated as identical. Based on the assigned profile for each TRS-PCR class, the tested strains were assigned to 40 TRS-PCR groups, differing in at least one TRS-PCR class. This way, 25 individual TRS-PCR groups, and 15 complexes were identified, to which at least two or more *P. aeruginosa* isolates were assigned.

In the next part of the doctoral dissertation, the results of differentiation of the studied collection of *P. aeruginosa* strains using the TRS-PCR numerical system were compared with the results of the VNTR-MLVA analysis obtained for these strains. 26 MLVA groups were identified for 63 *P. aeruginosa* isolates having a unique numerical allelic profile corresponding to the copy number of the VNTR sequence. Comparing the above methods, the assignment of numerical classes and groups of TRS-PCR differentiates clinical isolates more strongly compared to VNTR-MLVA, which is also indicated by the calculated values of Wallace's coefficient. Individual patients were assigned strain numbers or variants based on MLVA groups. This analysis was extended to include the specific numerical values of the TRS-PCR groups and has resulted in further diversification of the *P. aeruginosa* strain collection.

The new method of analysis of TRS-PCR profiles seems beneficial in the differentiation of clinical strains of *E. coli* and *P. aeruginosa* and shows a greater ability to differentiate isolates than the classical, averaged analysis of the band profiles. The applied numerical method proved that even slight genetic differences between bacterial strains could be detected.

Our research results suggest that MLST and TRS-PCR are effective methods for genotyping clinical *E. coli* strains and can be used in routine epidemiological surveillance and identification of *E. coli* transmission sources. It seems, however, that the numerical TRS-PCR method is in some cases more differentiating than MLST. Therefore, the numerical interpretation of TRS-PCR results proposed in this work can complement the MLST analyses.

The results of the research presented in the dissertation suggest that MLVA in combination with TRS-PCR are reproducible and highly differentiating methods of typing DNA of *P. aeruginosa* isolates from patients with cystic fibrosis. The new TRS-PCR methodology shows a greater differentiation power than the VNTR-MLVA technique. It can complement the MLVA scheme or be a primary, rapid, reproducible method for genotyping *P. aeruginosa* isolates. Applying the numerical method showed that detecting even minor genetic differences between bacterial strains was possible.

In conclusion, this dissertation proposes a new approach to interpreting TRS-PCR genotyping, in which each unique TRS-PCR profile is assigned a numerical value. If we want to look for similarities or carry out phylogenetic investigations, we can use the average TRS-PCR method (averaging the results of single typing). The numerical method, the sum of the results of single typings, allows the maximum differentiation of strains for epidemiological investigations, and the obtained numerical results are easy to interpret. The TRS-PCR band profile analysis method shows high differentiation power and reproducibility, and the numerical interpretation of TRS-PCR profiling results can be used as a complementary molecular tool to identify pathogenic strains in clinical trials and epidemiological investigations.