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RAPID DIFFERENTIATION OF BACTERIAL SPECIES BY HIGH RESOLUTION MELTING CURVE ANALYSIS

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Molecular based differentiation of various bacterial species is important in phylogenetic studies, diagnostics and epidemiological surveillance, particularly where unusual phenotype makes the classical phenotypic identification of bacteria difficult. Molecular approach based on the sequence of 16S ribosomal RNA gene analysis can achieve fast and reliable identification of bacteria. High resolution melting (HRM) curve analysis has been developed as an attractive novel technique for DNA sequence discrimination but it's application for bacteria differentiation has not been well studied yet. We have developed HRM assay for differentiation of sixteen pathogenic or opportunistic bacterial species. Amplified partial 16S ribosomal RNA gene region between 968 and 1401 positions (*E. coli* reference numbering) was subsequently used in high resolution melting curve analysis of PCR products for bacterial species differentiation. Sixteen bacterial species were simultaneously discerned by difference plot of normalized and temperatures shifted melting curves, without need for spiking of DNA, hetero-duplexing experiments or application of several primer pairs. High resolution melting curve analysis of duplex DNA is simple, fast and reliable tool for bacterial species differentiation and may efficiently complement phenotypic identification of bacteria.

Culture independent, molecular based differentiation of various bacterial species is important in many situations; in phylogenetic studies of various microbial communities [1], as well as in various clinical settings [2]. Also, reliable identification and typing of bacteria is also needed for an epidemic surveillance [3, 4]. In microbiology laboratories, traditional methods for bacterial identification rely on phenotypic analyzes, which under optimal growth conditions correctly identify bacteria with known phenotype. On the other hand, when known bacteria are presented with unusual phenotype phenotypic identification may be difficult and error prone [5]. In these situations, molecular methods based on DNA sequence analysis could achieve fast and reliable identification of bacteria, and nonviable and dead bacteria could be detected, further fastidious and difficult-to-cultivate bacteria could be identified as well [5, 6].

In particular, PCR based strategies, targeting specific genetic regions [3, 4, 7] or by amplification of ribosomal genes by broad range primers [2, 5] have proven fast and reliable methods for molecular differentiation of bacteria. However for simultaneous differentiation of several bacterial species, methods targeting specific genetic regions require design of many specific primers or probes and are time consuming. On the other hand current methods using amplification of ribosomal genes using broad range primers need post-PCR processing step, with exposing PCR products, which increases risk of contamination.

Ribosomal genes are most often used genetic regions for bacterial phylogenetic relationship studies, since they are ubiquitous, and in evolutionary sense they are operating as molecular chronometer [8]. The 16S ribosomal RNA gene has been suggested as the most suitable for use in phylogenetic studies [9]. The gene has multi-domain structure with conserved and variable regions and is large enough for statistically valid measurements of phylogenetic relations [10], hence providing a basis for molecular differentiation of bacteria.

High resolution melting (HRM) curve analysis of DNA duplex is a method for DNA sequence discrimination; it is close-tube method and could be used directly after amplification without exposing PCR products [11]. The method has been initially introduced as genotyping method with continuous detection of fluorescent signal generated during melting of amplicons after real time PCR in a temperature gradient [12]. Differences between PCR products of the same size are displayed by the shape of their melting curves, which depend on base distribution, proportion of GC bases and concentration of PCR products [13]. Application of fluorescent DNA saturation dyes in HRM curve analysis, such as LC Green I dye, has further simplified and extended usefulness of HRM curve analyses, as marked primers or probes are no longer needed [11]. Also, LC Green I DNA dye has been reported advantageous over to conventional SYBR Green DNA dye, since it does not redistribute within DNA duplex during melting process from melted to coiled regions of DNA and it is

not prone to inhibit amplification at saturating concentrations [11, 14].

So far a limited number of studies evaluated the HRM curve analysis as a method for discrimination among bacterial species [15, 16], and suggested HRM as potential cost effective approach for bacterial differentiation [16]. However, approach introduced by Cheng et al. [16] still requires hetero-duplexing between the PCR products of the tested and reference bacterial species or the 2nd real-time PCR targeting a different region of the 16S ribosomal RNA gene for identification of closely related bacteria species. We hypothesized that HRM is particularly suitable for a single broad range primer pair amplification approach that could be used for simultaneous differentiation of several different bacterial species.

In this study, we therefore optimized PCR with a single broad range primer pair targeting 16S ribosomal RNA gene V6-V8 region between 968 and 1401 positions (*E. coli* reference numbering), with LC Green I DNA dye for subsequent HRM analysis of a panel of sixteen bacterial species which displayed different degrees of DNA sequence diversity, ranging from 69.7 up to 98.9% sequence identity in amplified 16S ribosomal RNA gene region.

MATERIALS AND METHODS

Bacterial strains and DNA isolation. A panel of fifteen type strains with ATCC numbers in parentheses and one clinical isolate was used in this study: *Staphylococcus epidermidis* (clinical isolate), *Escherichia coli* (ATCC 25922), *Bacteroides fragilis* (ATCC 25285), *Clostridium perfringens* (ATCC 13124), *Peptostreptococcus anaerobius* (ATCC 27337), *Eubacterium lentum* (ATCC 43055), *Salmonella typhimurium* (ATCC 14028), *Shigella flexneri* (ATCC 12022), *Enterobacter cloacae* (ATCC 13047), *Serratia marcescens* (ATCC 13880), *Citrobacter freundii* (ATCC 8090), *Yersinia enterocolitica* (ATCC 9610), *Proteus mirabilis* (ATCC 43071), *Streptococcus pyogenes* (ATCC 19615), *Klebsiella pneumoniae* (ATCC 13883) and *Enterococcus faecalis* (ATCC 29212).

Bacteria were plated on sheep blood agar, incubated aerobically, or anaerobically at 37°C for 24 to 48 h, than 5 to 7 colonies was used for DNA extraction. DNA was extracted and purified from colonies with tissue modified procedure of Qiamp DNA mini kit (Qiagen, Germany): colonies were picked up from a plate, suspended in 180 µl of ATL buffer and incubated with 20 µl of proteinase K (1 mg/ml) for 35 min at 56°C, without lysozyme or other cell wall degrading enzymes, than tissue protocol was followed. DNA concentration and purity was determined by photometry at 260 and 280 nm wavelengths.

PCR amplification and DNA sequencing. Extracted DNA was used as a template for 20 µl PCR reactions. PCR was preformed with 1 U of *Taq* polymerase (Fermentas, Lithuania), 2.5 mM $MgCl_2$, 1x PCR buffer with ammonium sulphate, 0.4 mM of each deoxynucleoside triphosphate, deionizied and autoclaved water, 0.5 µM of each primer f-968: 5'-AACGCGAAGAACCTTAC-3' and r-1401: 5'-CGGTGTGTACAAGACCC-3' amplifying V6-V8 portion of 16S ribosomal RNA [17]. The reaction was performed in a thermocycler (Biometra, Germany) as follows: 3 min at 95°C, 35 cycles of 40 s at 94°C, 40 s at 56°C, 40 s at 72°C, and 3 min at 72°C, and amplicons of 430 nucleotides were checked by agarose (1.5%) gel electrophoresis. Amplicons were purified with the QIAquick PCR purification kit (Qiagen, Germany) according to manufacturer's instructions and were sent for sequencing to Macrogen, Inc, Korea (www.macrogen.com). Derived sequences were first compared with sequences available in the GeneBank, EMBL, and DDBJ databases using BLAST algorithm [18] through the National center for biotechnology information server (http://www.ncbi.nlm.nih.gov), and all positions showing differences from the best scoring reference sequences were visually inspected in the electropherogram and corrected if necessary. Then derived sequences were aligned with the Clustal X software package [19] and sequence identity matrix (Table 1) was constructed with the BioEdit software package (www.mbio.ncsu.edu/BioEdit/).

Sequence accession numbers. Sequences derived from this study were deposited in the EMBL nucleotide sequence database with accession numbers as follows: FM207087 to FM207089 and from FM207091 to FM207103. Six out of 16 sequences deposited to the EMBL from our study have to the best of our knowledge not been previously published: FM207094, FM207099, FM207096, FM207101, FM207103 and FM207100.

Real time PCR and high resolution melting curve analyses. Real time PCR and high resolution melting analyses were performed on the Roche LightCycler 480 instrument (Roche, Germany) in a 10 µl reactions with 0.25 U of GoTaq hot start polymerase (Promega, USA), 2 mM $MgCl_2$, 0.2 mM of each deoxynucleoside triphosphate, deionized and autoclaved water, 1X LC Green I (Idaho Technology, USA), 1x PCR buffer with KCl, 0.2 µM of each primer f-968 and r-1401, amplifying V6-V8 portion of 16S rRNA gene [17], and 1 µl of bacterial DNA was added. Reactions were performed as follows: 2 min at 95°C, 40 cycles of 10 s at 95°C, 10 s at 58°C, and 10 s at 72°C. Each PCR analysis was performed in duplicate. For HRM curve analyses, amplicons were denatureted at 95°C for 60 s, renaturated at 40°C with ramp rate of 2.2°C/s and 60 s hold, than heated to 75°C with ramp rate 1°C/s and 1 s hold, and melted from 70 to 95°C with ramp rate 0.03°C/s and 35 fluorescence acquisitions per Celsius degree. Generated raw fluorescence curves were analyzed with LightCycler 480 software package. First absolute quantification was performed to check the amplification curves and the crossing point (Cp) value. For discerning of

Table 1. Sequence identity matrix for bacterial strains used in this study with pair-wise proportion of the sequence identity

Species	*S. marcescens*	*C. freundii*	*E. coli*	*S. flexneri*	*E. cloacae*	*S. typhimurium*	*K. pneumonia*	*Y. enterocolitica*	*P. mirabils*	*P. epidermidis*	*C. perfringens*	*P. anaerobius*	*E. lentum*	*S. pyogenes*	*E. faecalis*	*B. fragilis*
S. marcescens	ID	0.979	0.944	0.946	0.959	0.961	0.952	0.939	0.941	0.816	0.765	0.767	0.758	0.773	0.785	0.714
C. freundii	0.979	ID	0.939	0.941	0.939	0.956	0.933	0.931	0.924	0.802	0.769	0.761	0.762	0.776	0.788	0.715
E. coli	0.944	0.939	ID	0.989	0.962	0.966	0.93	0.924	0.929	0.811	0.77	0.76	0.758	0.778	0.782	0.719
S. flexneri	0.946	0.941	0.989	ID	0.969	0.969	0.938	0.931	0.926	0.808	0.771	0.765	0.764	0.78	0.79	0.715
E. cloacae	0.959	0.939	0.962	0.969	ID	0.967	0.967	0.932	0.929	0.817	0.75	0.765	0.748	0.778	0.788	0.705
S. typhimurium	0.961	0.956	0.966	0.969	0.967	ID	0.945	0.931	0.926	0.821	0.772	0.76	0.772	0.788	0.792	0.718
K. pneumonia	0.952	0.33	0.93	0.938	0.967	0.945	ID	0.923	0.92	0.807	0.742	0.752	0.74	0.77	0.782	0.697
Y. enterocolitica	0.939	0.931	0.924	0.931	0.932	0.931	0.923	ID	0.959	0.826	0.769	0.77	0.762	0.781	0.79	0.72
P. mirabils	0.941	0.924	0.929	0.926	0.929	0.926	0.92	0.959	ID	0.839	0.775	0.773	0.758	0.791	0.788	0.718
P. epidermidis	0.816	0.802	0.811	0.808	0.817	0.821	0.807	0.826	0.839	ID	0.795	0.828	0.773	0.831	0.848	0.725
C. perfringens	0.765	0.769	0.77	0.771	0.75	0.772	0.742	0.769	0.775	0.795	ID	0.883	0.79	0.823	0.822	0.744
P. anaerobius	0.767	0.761	0.76	0.765	0.765	0.76	0.752	0.77	0.773	0.828	0.883	ID	0.78	0.83	0.827	0.724
E. lentum	0.758	0.762	0.758	0.764	0.748	0.772	0.74	0.762	0.758	0.773	0.79	0.78	ID	0.785	0.792	0.723
S. pyogenes	0.773	0.776	0.778	0.78	0.778	0.788	077	0.781	0.791	0.831	0.823	0.83	0.785	ID	0.893	0.759
E. faecalis	0.785	0.788	0.782	0.79	0.788	0.792	0.782	0.79	0.788	0.848	0.822	0.827	0.792	0.893	ID	0.744
B. fragilis	0.714	0.715	0.719	0.715	0.705	0.718	0.697	0.72	0.718	0.725	0.744	0.724	0.723	0.759	0.744	ID

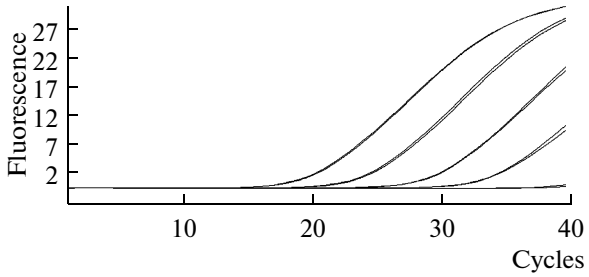

Fig. 1. Amplification curves for ten folds serial dilution of *E. coli* genomic DNAs with detection limit of 0.0002 ng/μl. Exponential growth of fluorescence signal is observed for each curve. Base line is a non-template control. Each sample was amplified in duplicate.

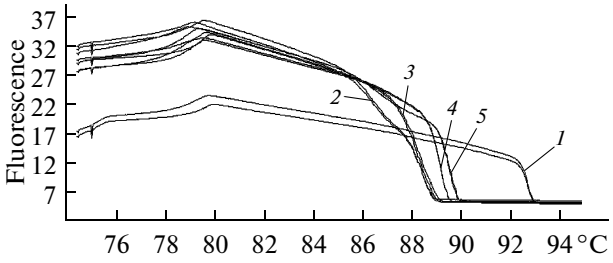

Fig. 2. Melting curves using raw fluorescence data. *Eubacterium lentum* (*1*), with melting transition at high temperature is clearly differentiated from the group of undiscernible bacteria; *Staphylococcus epidermidis* (*2*), *Peptostreptococcus anaerobius* (*3*), *Bacteroides fragilis* (*4*), *Enterobacter cloacae* (*5*). Each sample is presented in duplicate.

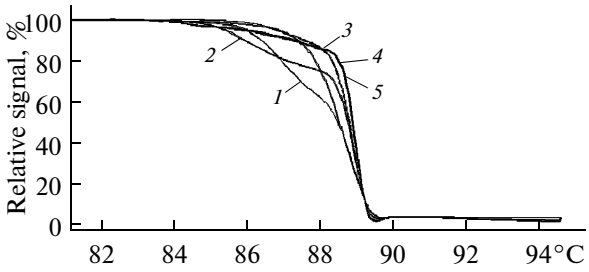

Fig. 3. Normalized fluorescence curves with 10% temperature shift. Examples for *S. epidermidis* (*1*), *E. cloacae* (*2*) and *B. fragilis* (*4*), with broad melting transition, *P. anaerobious* (*3*), *E. lentum* (*5*), with narrow melting transition. Each sample is presented in duplicate.

bacteria by sequence variation, gene scanning analysis was performed. Fluorescence curves were normalized and temperature shifts were done at 10% level of normalized fluorescence. In some cases, different temperature shifts were done if differentiation was not possible at 10% temperature shift. For discerning *E. cloacae* from *S. marcescens*, and *P. mirabilis*, temperature shift was done at 0% of normalized fluorescence, and for discerning *S. marcescens* and *P. mirabilis* temperature shift at 70% of normalized florescence was done. After normalized and temperature shifted melting curves were obtained, first negative derivative of fluorescence over temperature was calculated (–dF/dT vs. T), and fluorescence was presented as melting peaks with calculated melting temperatures (Tm) [13]. Than, in order to facilitate visual curve differentiation, fluorescence difference plots were created, by subtracting negative derivative of fluorescence for each species from negative derivative of fluorescence of *E. coli*, which was selected as reference base curve and plotted against temperature [11].

RESULTS AND DISCUSSION

In the present study, we amplified a V6-V8 portion of 16S ribosomal RNA gene region between 968 and 1401 positions (*E. coli* reference numbering) in 16 bacterial isolates for subsequent HRM curve analysis for simultaneous differentiation of bacterial species. We first determined detection limit of the HRM assay with *E. coli* genomic DNA serial ten fold dilutions, which as shown in Fig. 1, was up to 0.0002 ng/μl of bacterial DNA. We also checked reproducibility of the HRM assay with three independent amplifications for each bacterium from the 16 bacteria panel analyzed in this study and only minor differences were observed within replicates of species difference curves, indicating low inter-run variability.

When performed HRM curve analysis, raw melting data were generated during melting process. Raw melting data enabled only limited differentiation of bacterial species, i.e. melting curve of *E. lentum* is distinguished from others analyzed due to high temperature melting transition, the rest shown on Fig. 2, including *S. epidermidis*, *P. anaerobius*, *E. cloacae*, and *B. fragilis,* are indiscernible. In order to achieve better differentiation of bacterial species, raw fluorescence data should be normalized to join together curve shoulders and obtain relative values of fluorescence as percentage of the difference between minimum and maximum fluorescence values for each reaction [20]. In addition to normalization, temperature shifts of melting curves can improve differentiation and enables to distinguish even small genotype differences such as single nucleotide polymorphisms (SNPs) [21]. We performed normalization of our raw fluorescence curves by first defining linear base lines before and after melting transitions of each sample, and by normalization of fluorescence intensity to values between 0 and 100%. In addition we performed temperature shifts to overlay the curves at 10% of normalized fluorescence (Fig. 3). Temperature shifts arrange melting curves positions on a temperature axis in a way to show relative temperature differences between curves rather than absolute temperatures [11]. Fig. 3, shows examples of normalized and temperature shifted curves for *S. epidermidis*, *E. cloacae*, *B. fragilis*, *P. anaerobious*, *E. lentum* which were all easily distinguished from each other by melting curve shape. We therefore suggest that normalization and temperature shift is absolutely necessary step in HRM raw data anal-

ysis for accurate differentiation between bacterial species. Using normalization and temperature shift of raw data we were able to simultaneously distinguish 16 different bacteria species into nine different groups. The first group included *S. epidermidis* and *K. pneumoniae*, the second group included *C. perfringens*, the third group included *E. faecalis*, *Y. enterocilitica* and *P. mirabilis*, the fourth group included *E. cloacae*, *B. fragilis*, *E. coli*, and *S. flexneri*, the fifth group included *S. marcescens*, the sixth group included *S. pyogenes*, the seventh group included *S. typhimurium*, the eighth group included *C. freundi* and *P. anaerobious,* the nineth group included *E. lentum,* however we were not able to distinguish bacteria from each other within a given group. Therefore, we were able to differentiate only five bacterial species out of sixteen; *C. perfringens*, *S. marcescens* and *S. pyogenes* and *S. typhimurium* and *E. lentum*.

The normalized and temperature shifted melting curve data from the same PCR amplification and HRM run could be further analyzed by calculating negative derivative of fluorescence over temperature (–dF/dT vs. T), thus displaying melting temperatures peaks, expressed as Tm values, which in theory could provide simple differentiation of bacterial species. Using calculated Tm values, we were able to clearly distinguish eight groups of bacteria, however only four groups included a single bacterial species; *E. lentum*, *E. cloacae*, *B. fragilis* and *C. perfringens*. The rest of the groups were not able to distinguish the bacteriae within a given group due to the overlapping Tm (Table 2). Therefore following bacteria were not distinguished from each other: *C. freundi*, *S. epidermidis*, *P. anaerobious* in the first group, *E. faecalis, Y. enterocolitica*, *S. marcescens*, *S. typhimurium* and *P. mirabilis* in the second group, *S. pyogenes and K. pneumoniae*, in the third group and *S. flexneri* and *E. coli* in the fourth group (Table 2). *E. coli* and *S. flexneri* are however, considered as the same species with 98.9% DNA sequence homology (Table 1). Figure 4 shows examples of melting peaks calculated by negative derivative of fluorescence over temperature (–dF/dT vs. T) for *P. mirabilis, Y. enterocilitica, S. typhimurium, and E. lentum*. Based on the mealting peak calculation we were able to clearly distinguish only *E. lentum* from the rest of three bacteria shown in Fig. 4. We believe melting peak calculation of normalized data in HRM analysis only slightly improve bacterial species discrimination. Multi-copy organization of 16S ribosomal RNA genes in bacterial genomes with inter-operon variability [22] is probably reason why many different amplicons with slight sequence differences are produced during PCR reaction when same primers are used for amplification of 16S ribosomal RNA gene segment. Many amplicons slightly different in sequence, generated several hetero-duplexes during HRM and created complex melting patterns (Fig. 4) with asymmetric melting process and cumulative temperature peaks, similar as observed when melting longer heterozygous amplicons from diploid human genome [23].

Table 2. Average melting temperatures (Tm) expressed as °C from three independent experiments for bacterial species as first negative derivative of fluorescence over temperature (–dF/dT vs. T) with standard deviations (STD)

Species	Tm	STD
Staphylococcus epidermidis	88.018	0.30
Escherichia coli	89.555	0.05
Bacteroides fragilis	89.048	0.40
Clostridium perfringens	87.837	0.40
Peptostreptococcus anaerobius	88.162	0.08
Eubacterium lentum	92.272	0.30
Salmonela typhimurium	88.987	0.05
Shigella flexneri	89.495	0.08
Enterobacter cloacae	89.438	0.04
Serratia marcescens	88.787	0.09
Citrobacter freundii	88.165	0.05
Yersinia enterocolitica	88.933	0.12
Proteus mirabilis	88.868	0.04
Streptococcus pyogenes	88.518	0.03
Klebsiella pneumoniae	88.453	0.09
Enterococcus faecalis	88.963	0.09

A combination of two approaches for DNA melting curve data analysis performed in our study, first based on normalization and temperature shift and second based on Tm calculation of normalized and temperature shifted fluorescence, could clearly distinguish together only seven out of sixteen bacterial species analyzed in our study.

In order to further improve differentiation we performed further analysis of normalized and temperature shifted melting curve data by creating difference plots (Fig. 5). For this purpose, simultaneous fluorescence difference curves were created by subtracting first negative derivative of fluorescence (–dF/dT) curve for each normalized and temperature shifted bacterial curve

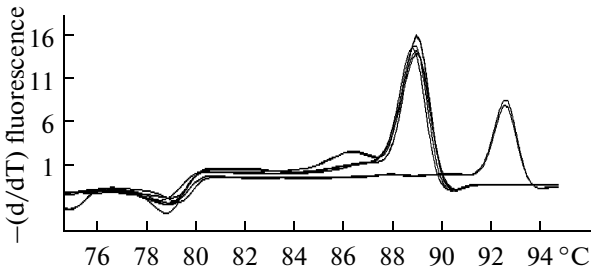

Fig. 4. Melting peaks (–dF/dT vs. T) over temperature. Examples for *P. mirabilis*, *Y. enterocilitica, S. typhimutium*, which are clustred together and could not be separated by melting peaks, further right is clearely separated mealting peak for *E. lentum*. Each sample is presented in duplicate.

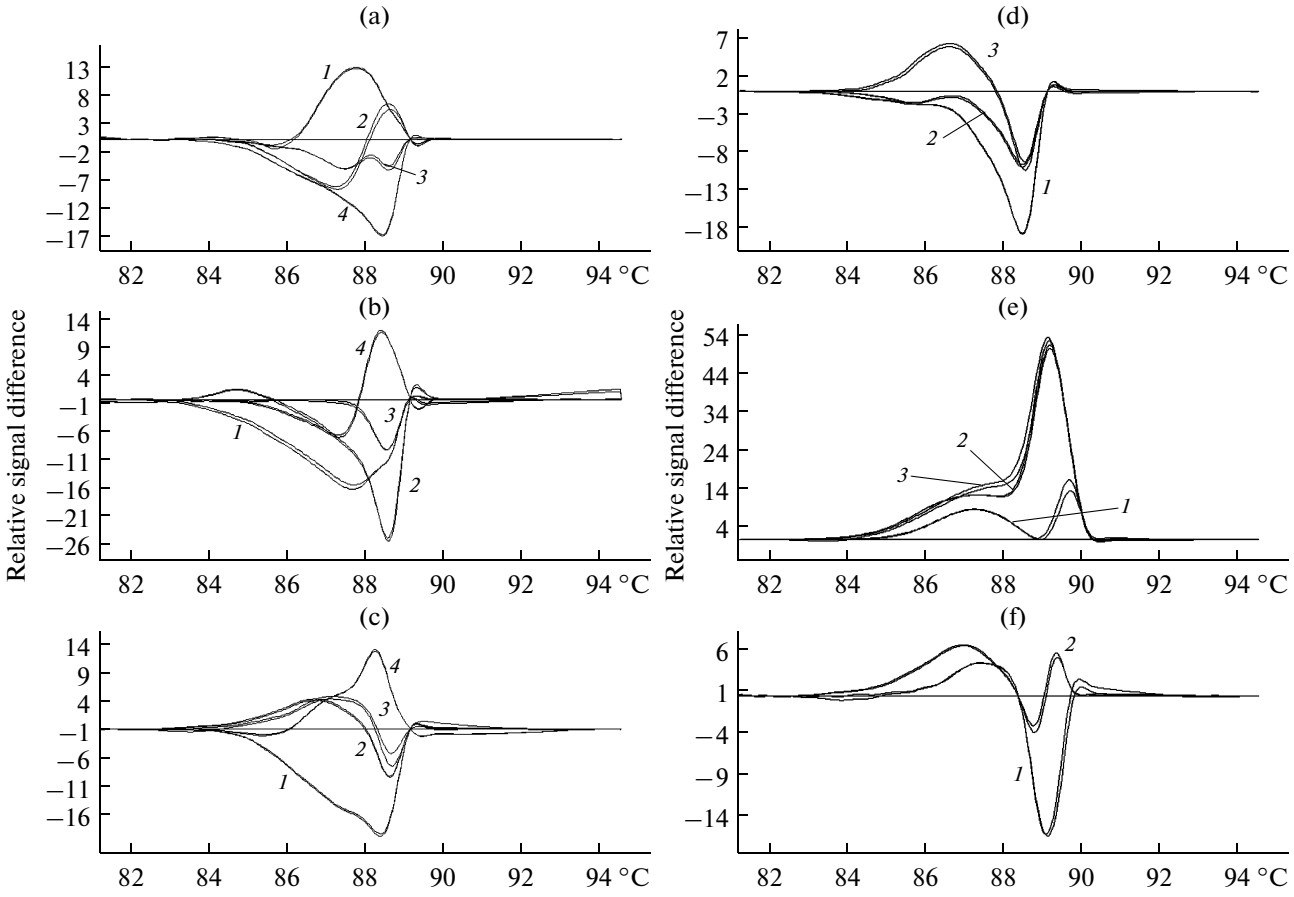

Fig. 5. Difference plot for (a) *S. epidermidis* (*1*), *P. anaerobious* (*2*), *C. perfringens* (*3*) and *B. fragilis* (*4*); (b) *S. typhimurium* (*1*), *E. lentum* (*2*), *S. flexneri* (*3*), and *C. freundii* (*4*); (c) *S. pyogenes* (*1*), *P. mirabilis* (*2*), *S. marcescens* (*3*), and *K. pneumoniae* (*4*), (d) *E. faecalis* (*1*), *Y. enterocolitica* (*2*) and *E. cloacae* (*3*), (e) *E. cloacae* (*1*), *S. marcescens* (*2*), and *P. mirabilis* (*3*). The *E. cloacae* is discernible from non-discernible *S. marcescens* and *P. mirabilis* with 0% temperature shift; (f) *Serratia marcescens* (*1*) and *P. mirabilis* (*2*) curves, with temperature shift of 70% are discernible. The *E. coli* was selected as reference species for plotting graph, and is represented with base line. Each species is presented in duplicate.

from *E. coli* base curve (linear) and plotted against temperature. The *E. coli* curve was arbitrarily selected as reference base curve for fluorescence difference plots, and by using *E. coli* as base curve it was easily to distinguish all 16 different bacteria species simultaneously, including the differentiation of *E. coli* from *S. flexneri* (Fig. 5b), despite their 98.9% sequence similarity (Table 1). For three pairs of most similar bacterial species, *E. cloacae* and *S. marcescens* pair, for *E. cloacae* and *P. mirabilis* pair, and for *S. marcescens* and *P. mirabilis* pair (Figs. 5a–5d), the difference plots were most difficult to be distinguished and therefore temperature shift was optimized. In addition to commonly used 10% temperature shift, 0 and 70% shifts were applied. Differentiation of *E. cloacae* from *P. mirabilis* and *S. marcescens* was most clearly achieved with 0% temperature shift (Fig. 5e). In contrast, differentiation of *P. mirabilis* and *S. marcescens* was most easily achieved with 70% temperature shift (Fig. 5f).

Taken together, difference plot with pre-selected base curve indeed enabled clear differentiation of all 16 bacterial species from each other, which in our experience makes this approach as the most suitable for bacteria differentiation. Also, we believe, melting peaks based differentiation could be a complement approach to difference plots, although further studies on heteroduplex formation are needed. The idea of using HRM analysis for bacteria differentiation has been initially introduced by Odell and coworkers [15]. They were able to differentiate three closely related *Mycobacterium* species (*M. chelonae*, *M. abscessus* and *M. immunogenum*) based on a few *hsp* 65 gene polymorphisms. The Odell study proved HRM analysis as an attractive approach for bacterial species differentiation, and warranted differentiation of more comprehensive panel of bacteria. In the initial study for bacterial species differentiation with HRM curve analysis of partial 16S ribosomal gene amplicons, Cheng and colleagues were able to distinguish 25 different bacterial species with high accuracy;

however only for less than half of bacterial species analyzed they were able to use simple 1-step post-PCR high-resolution melting analysis protocol [16]. To distinguish among the rest of bacteria species analyzed including most closely related bacteria species such as a group of enteric bacteria, they needed to perform hetero-duplexing between the PCR products of the tested and reference bacterial species or the 2nd real-time PCR targeting a different region of the 16S rRNA gene [16]. In this study we show that using only a single pair of primers for amplification of 16S rRNA gene region between 968 and 1401 positions (*E. coli* reference numbering) is particularly useful for HRM analysis for bacterial discrimination. For this region as compared to other variable regions of the 16S ribosomal RNA gene, greater heterogeneity among bacterial species has been shown [24]. This genetic region of about 430 base-pairs has been widely used for profiling of diverse bowel bacterial communities [25, 26] however to the best of our knowledge; it has not been used yet for HRM curve analysis. Using our approach we were able to distinguish between 16 different bacterial species only by close-tube PCR reaction and HRM analysis without additional hetero-duplexing or spiking procedures required. In order to avoid misidentification, phenotypically well described bacterial ATCC type strains were used in this study. We believe bacterial type strains represent link between phylogeny and taxonomy, and could serve as standards when HRM curve analysis approach is applied for routine identification in microbiology laboratory.

For this study, we intentionally selected a panel of bacteria which displayed different degrees of DNA sequence diversity, ranging from 69.7% for *K. pneumoniae* and *B. fragilis* pair to 98.9% for *S. flexneri* and *E. coli* pair (Table 1). Since all sixteen bacterial species were simultaneously differentiated from each other based on unique HRM melting profiles, analyzed as difference plots (Fig. 5), we believe our HRM approach shows high fitness of the method.

In addition to species identification, HRM curve analysis based genotyping of bacterial strains is a challenge as well. Published studies for genotyping of *Bacillus anthracis* [3] and *Campylobacter jejuni* [4], based on amplification of the variable number of tandem repeats in genome, and subsequent HRM curve analysis were in a good agreement with length polymorphism of tandem repeats. However, for several close related strains, differing in a single nucleotide polymorphism, HRM curve analysis failed to differentiate genotypes [3, 4].

In this study we have shown that an approach for DNA sequence discrimination, HRM curve analysis, applied to partial bacterial 16S rRNA sequence after PCR amplification, is rapid and efficient method for differentiation of bacterial species. A new HRM scheme for simultaneous bacterial species differentiation presented in this study could efficiently complement phenotypic identification of known bacteria when displaying uncommon phenotype or alternatively could enhance identification of new bacterial species. For routine identification, data base with type strains as standards should be created. For larger number of species in the data base, it may be necessary to analyze several different regions of 16S ribosomal gene.

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