Research article/Raziskovalni prispevek

MOLECULAR IDENTIFICATION OF SEVERAL CLINICALLY IMPORTANT BACTERIA INCLUDING ENTEROBACTERIA BY PARTIAL 16S RIBOSOMAL RNA GENE SEQUENCE COMPARISON

MOLEKULARNA IDENTIFIKACIJA KLINIČNO POMEMBNIH BAKTERIJ, VKLJUČNO ENTEROBAKTERIJ S PRIMERJAVO DELNEGA NUKLEOTIDNEGA ZAPOREDJA GENA ZA 16S RIBOSOMSKO RNK

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Abstract

Background *The correct identification of bacterial isolates is paramount for the correct diagnosis and treatment of patients. The standard approach for bacterial identification relies on phenotypic tests, which are often time-consuming and unreliable when identifying atypical strains. We instead hypothesized a molecular approach; fast and reliable identification could be achieved by comparing a 16S ribosomal RNA gene sequence with publicly available sequences.*

Methods *In the present study, DNA from seventeen bacterial species was extracted and amplified by PCR, and then a partial 16S ribosomal RNA gene sequence was obtained and compared with sequences deposited in public DNA sequence databases; EMBL/GeneBank/DDBJ and RDP-II.*

Results *Bacterial species, belonging to several families; Coriobacteriaceae, Bacteroidaceae, Clostridiaceae, Staphylococcaceae, as well as three out of nine Enterobacteriaceae were specifically identified by partial 16S ribosomal RNA gene sequence to the species level. For six other species of enteric bacteria specific identification of species failed, since the results of genotypic identification yielded several species.*

Conclusions *We conclude identification of bacteria with partial 16S ribosomal RNA gene sequence is an efficient complement to phenotypic identification, except for enteric bacteria.*

Key words *molecular identification; bacteria; 16S rDNA sequences*

Izvleček

Za učinkovito diagnostiko bakterijskih okužb in zdravljenje je potrebna hitra in natančna identifikacija bakterije. Običajen postopek je fenotipska identifikacija, ki je pogosto zahtevna in nenatančna za atipične izolate. V naši študiji smo predpostavili, da je možno identificirati bakterije s primerjavo delnih nukleotidnih zaporedij genov, ki kodirajo 16S ribosomsko RNK s primerjavo z nukleotidnimi zaporedji v javno dostopnih podatkovnih bazah za zaporedja DNK: EMBL, GenBank, DDBJ in RDP-II. Še posebej nas je zanimala možnost identifikacije enterobakterij, saj mnogi avtorji navajajo, da te skupine bakterij ni

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moč ločiti na osnovi zaporedij genov 16S ribosomske RNK. Za izvedbo študije smo pridobili 15 tipskih sevov in dva klinična izolata iz zbirke Zavoda za zdravstveno varstvo Maribor, katerih rutinska fenotipska identifikacija je težavna. Iz bakterij smo izolirali DNK ter z verižno reakcijo s polimerazo (PCR) pomnožili variabilen odsek gena za 16S ribosomsko RNK. Pridelke PCR smo očistili in poslali na sekveniranje s prednjim začetnim oligonukleotidom v podjetje Macrogen, Koreja (www.macrogen.com). Pridobljena nukleotidna zaporedja DNK smo nato z dvema iskalnima algoritmoma; BLAST, v bazah GeneBank/EMBL/DDBJ, ter z algoritmom Sequence match v bazi RDP-II primerjali z vloženimi nukleotidnimi zaporedji, za morebitno identifikacijo vrste. Specifično smo uspeli identificirati vrste: Staphylococcus epidermidis, Bacteroides fragilis, Clostridium perfringens, Peptostreptococcus anaerobius, Eggerthella lenta, Enterobacter cloacae, Yersinia enterocolitica, Proteus mirabilis, Streptococcus pyogenes, Enterococcus faecalis. Medtem ko Escherichia coli, Serratia marcescens, Shigella flexneri, Klebsiella pneumoniae, Citrobacter freundii nismo uspeli nedvoumno identificirati, čeprav je klinično nujno. Vendar menimo, da je opisan genotipski pristop za identifikacijo vrste bakterije učinkovito dopolnilo fenotipski identifikaciji, še posebno pri identifikaciji atipičnih izolatov.

Ključne besede *molekularna identifikacija; nukleotidno zaporedje; bakterije*

Introduction

Accurate identification of bacterial isolates is essential task for clinical microbiology laboratories. Currently, standard methods for bacterial identification rely on phenotypic methods. However, for many fastidious bacteria, phenotypic identification is not only difficult and time consuming, but the identification of species is difficult due to the phenotypic variability among strains belonging to the same species. In these situations, a molecular approach, based on DNA sequence analysis, could achieve identification.[1] Two PCR-based approaches are widely used for molecular bacterial identification: the first targets species or group specific genes,[2] while the second approach uses PCR amplification of ribosomal genes by broad range primers for bacteria detection and require a second post-PCR processing step for identification.[3]

Ribosomal genes are ubiquitous, multicopy genes, in evolutionary sense operating as molecular chronometers, hence reflecting bacterial phylogenetic relatedness.[4] The 16S ribosomal RNA gene has been proposed as most suitable for use in phylogenetic studies.[5] Partial sequences of 16S ribosomal RNA gene should be sufficient for the identification of species when compared with some longer, closely related sequence. In contrast, the entire 16S ribosomal RNA gene sequence is required for new species identification.[6, 7] Due to extensive use of 16S ribosomal gene sequences for phylogenetic studies, public domain databases have been established for sequences deposition and comparison.[8, 9] Indeed, comparisons between partial 16S ribosomal RNA gene sequences and sequences deposited in public domain DNA databases has proven an efficient complement to phenotypic methods, i.e. for gram-positive rods,[10] for identification of aerobic catalase-negative, gram-positive cocci,[11] for non fermenting gram negative bacteria,[12] for enteric bacteria, limited results have been reported.[13]

The aim of this study was to assess the feasibility of the amplification of partial 16S ribosomal RNA gene by a single broad range primer pair, and subsequent sequencing of PCR products for bacterial species identification. We were particularly interested in enteric bacteria identification.

Material and methods

Bacterial species and DNA isolation

As listed in Table 1, fifteen bacterial quality control type strains were obtained from the American type culture collection (ATTC); *Escherichia coli*, *Bacteroides fragilis*, *Clostridium perfringens*, *Peptostreptococcus anaerobius*, *Eggerthella lenta*, *Salmonella Typhimurium*, *Shigella flexneri*, *Enterobacter cloacae*, *Serratia marcescens*, *Citrobacter freundi*, *Yersinia enterocolitica*, *Proteus mirabilis*, *Streptococcus pyogenes*, *Klebsiella pneumoniae*, *Enterococcus faecalis* and two clinical isolates (*Prevotella* spp., and *Staphylococcus epidermidis* were obtained from clinical microbiology laboratory for this study. DNA was extracted and purified from colonies with tissue modified following the Qiamp DNA mini kit procedure (Qiagen): a colony was 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 minutes at 56 °C, without lizocyme or other cell wall degrading enzymes. Tissue protocol was then followed.

PCR and sequencing

Extracted DNA was used as a template for 20 µl PCR reactions. PCR was performed with 2U of *Taq* polymerase (Fermentas), 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'.[14] The reaction was performed in a thermocycler (Biometra) 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. PCR products of about 430 nucleotides were observed by agarose (1.5 %) gel electrophoresis. Ampli-

Table 1. *Bacterial strains used for this study, source, and molecular identification results with highest similarity score with compared sequences.*

Razpr. 1. *Bakterijski sevi, uporabljeni v študiji, vir in rezultati molekularne identifikacije z najvišjo vrednostjo ujemanja primerjanih zaporedij.*

Isolate number	Bacterial strain and phenotypic identification	Source	Identification by sequence alignment with BLAST algorithm in GeneBank, DDBJ, EMBL databases	Highest similarity score percentage with BLAST algorithm	Identification by sequence alignment with Sequence match algorithm in RDP database	Highest S_{ab} value with Sequence match algorithm
Številka izolata	Bakterijski sev in fenotipska identifikacija	Vir	Identifikacija s primerjavo zaporedij v skladu z BLAST algoritmom v GeneBank, DDBJ, EMBL bazah podatkov	Najvišja vrednost ujemanja z BLAST algoritmom (v odstotkih)	Identifikacija s primerjavo zaporedij v skladu z algoritmom za primerjavo zaporedij v RDP-bazi podatkov	Najvišja S_{ab} vrednost pri algoritmu za primerjavo zaporedij
1	*Staphylococcus epidermidis*	Clinical isolate Klinični izolat	*Staphylococcus epidermidis*	100	*Staphylococcus epidermidis*	1.00
2	*Prevotella spp.*	Clinical isolate Klinični izolat	*Bacteroides fragilis*	99	*Bacteroides fragilis*	0.44
3	*Bacteroides fragilis*	ATCC 25285	*Bacteroides fragilis*	99	*Bacteroides fragilis*	0.981
4	*Clostridium perfringens*	ATCC 13124	*Clostridium perfringens*	100	*Clostridium perfringens*	1.00
5	*Peptostreptococcus anaerobius*	ATCC 27337	*Peptostreptococcus anaerobius*	100	*Peptostreptococcus anaerobius*	1.00
6	*Eggerthella lenta*	ATCC 43055	*Eggerthella lenta*	100	*Eggerthella lenta*	0.995
7	*Escherichia coli*	ATCC 25922	*Escherichia coli, Shigella sonnei, Shigella flexneri, Shigella dysenteriae, Shigella boydii*	99	*Escherichia coli, Shigella flexneri, Shigella boydii, Shigella sonnei, Escherichia albertii, Escherichia fergusonii*	0.966
8	*Salmonella typhimurium*	ATCC 140028	*Salmonella enterica* sub. *Enterica,* ser. Paratyphi B, Paratyphi C	100	*Salmonella entericaca sup. enterica, ser. Typhimurium*	1.00
9	*Shigella flexneri*	ATCC 12022	*Shigella flexneri, Shigella boydii, Shigella sonnei, Shigella dysenteriae, Escherichia coli*	100	*Escherichia coli, Shigella boydii, Shigella flexneri, Shigella sonnei, Escherichia albertii, Escherichia fergusonii*	1.00
10	*Enterobacter cloacae*	ATCC 13047	*Enterobacter cloacae,*	100	*Enterobacter cloacae*	1.00
11	*Serratia marcescens*	ATCC 13880	*Serratia marcescens, Enterobacter* aerogenes, *Kluyvera cryocrescens*	100	*Serratia marcescens, Kluyvera cryocrescens*	1.00
12	*Citrobacter freundii*	ATCC 8090	*Citrobacter freundii, Klebsiella* oxytoca, *Raoultella terrigena*	100	*Citrobacter freundii, Klebsiella oxytoca,*	0.984
13	*Yersinia enterocolitica*	ATCC 9610	*Yersinia enterocolitica*	100	*Yersinia enterocolitica*	0.995
14	*Proteus mirabilis*	ATCC 43071	*Proteus mirabilis*	100	*Proteus mirabilis*	1.00
15	*Streptococcus pyogenes*	ATCC 19615	*Streptococcus pyogenes*	100	*Streptococcus pyogenes*	0.971
16	*Klebsiella pneumoniae*	ATCC 13883	*Klebsiella pneumoniae, Klebsiella variicola, Klebsiella oxytoca*	100	*Klebsiella pneumoniae, Klebsiella variicola, Klebsiella oxytoca*	1.00
17	*Enterococcus faecalis*	ATCC 29212	*Enterococcus faecalis, Enterococcus dispar*, Lactobacillus plantarum	100	*Enterococcus faecalis*	0.955

cons were purified with the QIAquick PCR purification kit (Qiagen) according to manufacturer's instructions and were sent for sequencing with forward primer f-968, and in cases of nucleotide ambiguity with reverse primer r-1401 to Macrogen, Inc. (www.macrogen.com).

16S ribosomal RNA gene sequence analysis

Determined sequences were compared with sequences available in GeneBank, EMBL, and DDBJ databases using the BLAST algorithm,[15] through the National center for biotechnology information server (www.ncbi.nlm.nih.gov) and with sequences available in Ribosomal database project (RDP-II) (http://rdp.cme.msu.edu), release 9.59, by use of Sequence match algorithm.[9] In order to assign isolate to a species, each derived sequence aligned by the BLAST algorithm, yielded at least 99 % similarity score with identified species in the BLAST search, and the highest S-ab value with identified species in the Sequence match search.

Results and discussion

In the present study, fifteen bacterial type strains were used and two clinical isolates were obtained from clinical microbiology laboratory (Table 1). DNA was then extracted and amplified between 968 and 1401 positions in the 16S ribosomal RNA gene (*Escherichia coli* reference numbering), giving 430 bp amplification product, and amplicons were purified and sequenced with f-968 forward primer and in case of ambiguous nucleotides with r-1401 reverse primer as well. Partial 16S ribosomal RNA gene sequences were obtained and checked, in order to ascertain whether identification of bacterial species would be possible through comparison with publicly available sequences with the BLAST algorithm in GeneBank/EMBL/DDBJ DNA databases, and with the Sequence match algorithm in the RDP-II database.

It is noteworthy that one difficulty of the sequence comparison approach for new species identification is that there are no generally accepted guidelines for comparison of sequence similarity for 16S ribosomal RNA gene sequence based identification. However some recommendations have been made: based on theoretical predictions of divergence, a difference of entire 16S ribosomal gene or protein coding region sequence > 0.5 % has been suggested as indicative for a new species within known genus.[16]

In this study, only the variable region of 16S ribosomal RNA gene was amplified by f-968 and r-1401 primer pair,[14] since greater heterogeneity has been displayed in this region among bacterial species, as compared with other variable regions of the gene.[17] This region of 16S ribosomal RNA gene has been widely used for the profiling of diverse bowel bacterial communities.[18, 19] Ideally, we would expect this region of 16S ribosomal sequence to differentiate among bacterial species, and on the other hand, for it to be conserved enough, for clustering of bacterial isolates of the same species. In addition, identification problems may arise from interoperon ribosomal sequence heterogeneity, which may also contribute to differences.[20] Since just a portion of variable 16S ribosomal RNA gene sequences was used for comparison, we expected only to identify existing species, while phylogenetic observations indicated new bacterial species were unlikely.[6, 7]

After the second step, the BLAST search, derived sequences were aligned with the Sequence match algorithm in the RDP-II database, which uses alignment information to calculate percent identity over all comparable positions and calculates binary association coefficient S_{ab}[21] in contrast to the BLAST algorithm, which reports only local identity over a fraction of sequence.[9] Sequence match algorithm should enable more reliable species identification. Search results with the BLAST algorithm in GeneBank/EMBL/DDBJ were able to specifically identify several enteric bacteria species: *Yersinia enterocolitica*, *Proteus mirabilis*, *Enterobacter cloacae*, and *Salmonella enterica*, although it was not identified as serovar Typhimurium in the BLAST search results. The same results were obtained with Sequence match algorithm in the RDP-II search (Table 1). *Shigellae* were indiscernible from each other and from *Escherichia coli*. This observation was expected, since *Escherichia coli* and *Shigella* are considered phylogenetically as the same species.[22] On the other hand, *Escherichia coli* isolate was specifically identified as *Shigella* or *Escherichia coli* with BLAST; however with the Sequence match algorithm two additional unclassified *Escherichia* species were identified (Table 1), indicating high intra-genus similarities of the *Escherichia* species, a phylogenetic property which is not clinically acceptable. However specific identification was not achieved for other enteric bacteria species. *Klebsiella pneumoniae* was not discerned from *Klebsiella variicola* and *Klebsiella oxytoca*, just as with *Escherichia*, indicating close intra-genus homogeneity. For *Serratia marcescens* and *Citrobacter freundii* classification to genus level failed (Table 1). *Citrobacter freundii* was not discerned from *Klebsiella oxytoca* and *Raoultella terrigena*. These observations are in accordance with phylogenetic observations, indicating that *Klebsiella* and *Raoultella* species are grouped with other representatives of *Enterobacteriaceae* genera, including with *Citrobacter freundii*.[23] *Serratia marcescens* was not discerned from *Enterobacer aerogenes* and *Kluyvera cryocrescescens*. This observation indicates the closer relationship between *Serratia marcescens* and *Kluyvera cryocrescescens*, however further phylogenetic analyses are needed for more decisive conclusions. The *Enterobacter* genus is phylogenetically interwined with members of several other genera, and *Enterobacter aerogenes* may be more closely related to *Serratia marcescens* than to the other *Enterobacter* species.[24] For enteric bacteria, the discriminatory power at species level of 16S ribosomal RNA gene sequences has been questioned, and it seems phenotypic approach yields higher level of discrimination.[13] Other studies reported failures in the 16S ribosomal gene sequence based approach to differentiating enteric bacteria,[1, 25] however in this study; we were able to delineate several enteric bacteria by partial 16S ribosomal RNA gene sequence comparison with publicly available sequences. But for clinical use, molecular approach should be at least as discriminative as phenotypic tests.

Several other bacteria species, members of different phyla: *Staphylococcus epidermidis*, *Clostridium perfringens*, *Bacteroides fragilis*, *Peptostreptococcus anaerobious*, *Eggerthella lenta*, *Streptococcus pyogenes* were identified specifically with BLAST algorithm, as well as with the Sequence match algorithm (Table 1). The amplified region of the 16S ribosomal RNA gene is diverse enough for discrimination of these species. In addition, clinical isolate number two, identified by phenotypic approach as *Prevotella* spp. (Table 1), was specifically identified as *Bacteroides fragilis*, which exemplifies the need for an improved routine identification scheme. However, this discrepancy is not unusual, since both genera, *Prevotella* and *Bacteroides* are phenotypically and genotypically closely related, and previous phylogenetic analyses, based on 16S ribosomal gene sequence had

shown that several *Prevotella* species fall in the genus *Bacteroides*.[26] *Peptostreptococcus anaerobius* was specifically identified by the BLAST algorithm, as well as with the RDP-II search (Table 1). Isolate number six (Table 1), identified as *Eggerthella lenta* is bacterium belonging to the *Coriobacteriaceae* family, and has long been classified as *Eubacterium lentum*. Since the genus *Eubacterium* is phylogenetically and phenotypically diverse, *Eubacterium lentum* has been reclassified as a type species of *Eggerthella* genus, named *Eggerthella lenta* on the basis of 16S ribosomal gene sequence analysis.[27]

Specific identification was observed for *Streptococcus pyogenes* identification with the BLAST algorithm, and with RDP-II search results, indicating that the section 16S ribosomal gene used for this study has sufficient discriminatory power. Although we were able to identify *Streptococcus pyogenes* specifically, use of the 16S ribosomal RNA gene sequence has been reported ambiguously for *Streptococcus* species identification.[28] On the contrary, nonspecific identification was observed with the BLAST algorithm for *Enterococcus faecalis*, since it was additionally identified as *Enterococcus dispar* and *Lactobacillus plantarum*. However, the existence of *Enterococcus dispar* as a new species has been questioned, since it was identified only by partial 16S ribosomal RNA gene sequences of two strains.[28] Searching in the RDP-II, led to the identification of *Enterococcus faecalis* as well as *Lactobacillus plantarum*. This observation indicates a close relationship, and may be explained by the horizontal gene transfer between these two species.[29]

We propose, for routine identification, that the query sequence should be compared with the BLAST algorithm in GenBank/EMBL/DDBJ databases and with the Sequence match algorithm in RDP-II database. In case of ambiguous identification with both algorithms, yielding several different species, we propose searching for the most similar type strains, which can enable identification by subsequent direct comparison of genetic regions and/or phenotypic characteristics. Molecular identification results should be correlated with phenotypic identification and interpreted together.

In conclusion, in this study, we were able to identify several bacterial type strains, including three out of nine enteric bacteria, to the species level by only partial 16S ribosomal gene sequence. We believe that the identification of bacterial isolates by partial 16S ribosomal RNA gene sequence can complement phenotypic identification efficiently, not only providing the final identification of species, but serving also as an orientation point for additional genotypic or phenotypic identification proceedings, thus facilitating bacterial diagnosis.

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