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SPECIFIC DETECTION OF *Salmonella spp*. WITH MOLECULAR BIOLOGICAL TECHNIQUES

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ABSTRACT

A method for specific detection of *Salmonella* spp. was developed based on 16S rRNA targeted PCR (polymerase chain reaction). A method based on recognition of specific region of 16S rRNA gene was published previously by Lin and Tsen in 1996. The sequence analysis of recently determined small subunit ribosomal genes from *Salmonella* spp. showed however, that the 16SF1 and 16SIII primers are not suitable for specific amplification of all *Salmonella* strains. 16SF1 was modified and a new forward PCR primer was designed. The specificity of the method was tested using 87 *Salmonella* strains and 30 non-*Salmonella* strains of the family *Enterobacteriaceae* and compared with two other previously published methods and found to be 100% specific. The reverse primer was also labelled with a tetramethylrhodamine isothyiocianate fluorescent dye and successfully used as a probe in *in situ* experiments.

Key words: microbiology / bacteria / *Salmonella* / molecular biology / methods / molecular genetic / rRNA

SPECIFIČNO ODKRIVANJE SALMONEL Z MOLEKULARNO BIOLOŠKIMI TEHNIKAMI

IZVLEČEK

Razvili smo specifično metodo za odkrivanje salmonel, ki temelji na pomnoževanju dela gena za 16S rRNK v verižni reakciji s polimerazo (PCR). Lin in Tsen sta l. 1996 že objavila metodo, zasnovano na pomnoževanju gena za 16S rRNK. S primerjavo nedavno ugotovljenih nukleotidnih zaporedij manjših ribosomskih podenot pri bakterijah iz rodu *Salmonella* smo ugotovili, da začetna oligonukleotida 16SF1 in 16SIII ne prepoznata vseh salmonel. Zato smo razvili nov proti 3' koncu usmerjen začetni oligonukleotid in modificirali 16SF1 začetni oligonukleotid. Specifičnost naše metode smo testirali na 87 različnih sevih salmonel in 30-ih salmonelam sorodnih sevih družine *Enterobacteriaceae* ter jo primerjali z dvema objavljenima metodama. Ugotovili smo 100% specifičnost naše metode. Enega od začetnih oligonukleotidov smo označili s fluorescentnim barvilom in ga uspešno uporabili kot oligonukleotidno sondo v *in situ* hibridizaciji.

Ključne besede: mikrobiologija / bakterije / salmonele / molekularna biologija / metode / molekularna genetika / rRNA

INTRODUCTION

Members of the genus *Salmonella* are gram-negative, facultatively anaerobic, rod-shaped, non-spore-forming bacteria. They are usually motile, catalase-positive, oxidase and urease-negative, they reduce nitrates to nitrites and use citrate as the sole carbon source, and do not deaminate phenilalanine and ferment lactose. Phenotypically and phylogenetically the genus *Salmonella* and the whole family *Enterobacteriaceae* belongs to the γ-subclass of proteobacteria. All of the *Salmonella* serotypes belong to two species. *Salmonella bongori* contains less than 10 serotypes, which are extremely rare. More than 2500 remaining serotypes belong to *Salmonella choleraesuis* (Bergey's Manual of Determinative Bacteriology, 1995). Most of the *Salmonella* serotypes have been isolated from humans and warm blooded animals. The disease caused by this bacteria, salmonellosis, is one of the most common illnesses also in the developed countries, therefore a rapid and punctual detection is required.

Conventional methods for detecting and identifying this pathogen involve cultural, biochemical and immunological assays, that rely on phenotypic characterisation. These methods require selective enrichment and plating, which often take several days to complete and may alter phenotypic expression. Nucleic acid based methods avoid such problems and might enable considerably faster detection. Several polymerase chain reaction (PCR) methods for detecting *Salmonella* have been published utilising specific gene sequences as targets. Most of the enumerated methods, however, enable recognition of only certain number of serotypes or groups of serotypes (for example only motile *Salmonella*) and not of the whole genus.

The 16S rRNA approach offers certain advantages when compared to methods based on unknown randomly cloned fragments. A large collection of 16S rRNA sequences is already available and offers the possibility of comparative studies and primer design even before the actual start of the laboratory experimentation. If designed to recognise the rRNA molecule rather than DNA, the specific oligonucleotides can be used as RNA probes in hybridisation experiments too. The large number of targets per cell, compared to the number of the genes in a chromosome, can be used as an advantage.

MATERIAL AND METHODS

Isolation of genomic DNA and PCR amplification

1 ml of bacterial suspension or its dilution or one colony of bacterial cells resuspended in 100 μl sterile distilled water was centrifuged at 13000 x g for 5 min, washed with distilled water and centrifuged again. The pellet was mixed with 100 μl 0.125% sodium dodecylsulfate and 0.05M NaOH solution, incubated at 95°C for 15 min and stored at –20°C until use (Trkov *et al.*, 1998). PCR amplification was carried out using a temperature programme consisting of the initial denaturation of DNA (5 min at 94°C), 30 cycles of: denaturation for 15 sec at 94°C, primers annealing for 15 sec at 58°C and extension for 30 sec at 72°C, and the final extension (4 min at 72°C). The reactions were performed in a Gene Amp PCR System 2400 thermal cycler (Perkin-Elmer). The amplification products were analysed by electrophoresis in 0.8 – 1.2 % TBE-agarose (SeaKem LE agarose) gel (TBE: Tris-borate EDTA buffer) at 6 V/cm.

Sequence analysis. Computer programs included in ClustalW (Higgins *et al.*, 1991) and Phylip (http://evolution.genetics.washington.edu/phylip.html) were used for the phylogenetic analysis of the 16S rRNA sequences retrieved from the DNA databases via Entrez browser (http://www3.ncbi.nlm.nih.gov/Entrez/).

In situ **hybridization**

Bacterial cells were grown in I broth (17 g/l bacteriological peptone, 3 g/l yeast extract, 5 g/l NaCl) at 37°C under vigorous shaking. Cells were harvested at mid-logarithmic phase (optical density at 650 nm 0.9 – 1.0). The procedures of cell fixation, *in situ* hybridization, epifluorescence microscopy and photomicrography were described by Tepšič and Avguštin (1997). The reverse PCR primer was marked at 5'end with fluorescent dye tetramethylrhodamine isothyocianate (TRITC) (MWG Biotech, Ebersberg, Germany) and used as oligonucleotide probe. Intensity and clearness of the signal was compared with a broadly specific oligonucleotide probe EUB 338 also labelled with TRITC. Hybridization was carried out for 16 hours at 50°C. Microscopic slides (Superfrost plus slides, MJ Research, inc.) were rinsed with sterile distilled water for 45 min at the same temperature.

RESULTS AND DISCUSSION

Several molecular biology based methods for specific detection of *Salmonella* in foods were published in recent years (Sheu *et al*., 1998). Majority of these methods are based on polimerase chain reaction that allow rapid and selective identification of microorganisms. Widjojomodjo *et al*. (1991) used replication genes, Rhan *et al*. (1992) invasion associated *invA* gene, Aabo *et al*. (1993) random genomic fragment, Way *et al*. (1993) flagellin genes, Doran *et al*. (1993) *agfA* gene; Lin and Tsen (1996) 16S rRNA genes, Wodward and Kirwan (1996) *sefA* gene, Zhu (1996) 5S-23S rRNA spacer region, Bej *et al*. (1994) and Jonsen *et al*. (1993) genes for DNA binding proteins and Cohen *et al*. (1996) *fimA* gene for major fimbrial subunit. Most of the mentioned methods have in common one deficiency – they are not genus specific, but recognise only a certain number of serotypes.

Aabo *et al*. (1993) developed primers for genus specific detection of *Salmonella,* selected from a random 2.3 kilobase long chromosomal fragment of *Salmonella typhimurium*, which hybridized to 214 tested serovars. The specificity of ST11 and ST15 primers, selected from mentioned fragment, was confirmed using 146 *Salmonella* serovars and 86 non-*Salmonella* strains of the family *Enterobacteriaceae*. The specific 429 base pairs long PCR product was correctly identified in all but two *Salmonella* strains. No false positive reactions were observed. We used this method as a control method, since other researchers confirmed its sufficient specificity.

Lin and Tsen (1996) published a method based on specific recognition of part of the 16S rRNA gene. 53 *Salmonella* and 33 non-*Salmonella* strains were tested, and using 16SF1 and 16SIII oligonucleotide primers, no false-positive and false-negative reactions were observed. However, when more than 500 16S rRNA sequences of *Salmonella* and other related bacteria from γ-proteobacteria phylogenetic group were retrieved from the GeneBank database, the computer analysis of sequences showed that using 16SF1 and 16SIII were not suitable for specific amplification from all *Salmonella* strains. Mismatches were found in one third of known 16S rRNA *Salmonella* sequences when compared to sequences of Lin and Tsen PCR primers. The forward PCR primer (16SF1) was therefore appropriately modified and used as a reverse primer and a new forward PCR primer was developed (Trkov and Avguštin, submitted for publication). Furthermore, the method was optimised, in order to decrease time needed for amplification of the app. 400 base pair long fragment, to only one hour and 30 minutes comparing to Lin and Tsen method that lasted two hours. The specificity of the described method was tested on 87 *Salmonella* strains (31 different serotypes) and 30 non-*Salmonella* strains (from 7 different genera of the family *Enterobacteriaceae*) closely related to the members of the genus *Salmonella*. The method was found to be entirely specific, correctly identifying all

tested *Salmonella* strains and giving no false-positive results when testing other bacteria. On the contrary, primers from Lin and Tsen did not recognise three different *Salmonella* serotypes (Heidelberg, Weltevreden, Houten) but did incorrectly enable amplification of the sought product with three different strains of the species *Enterebacter cloacae*. The method of Aabo *et al*. using primers ST11 and ST15 (1993) was found to be equally specific as the procedure described in this paper.

The reverse PCR primer was labelled at 5' end with fluorescent dye (TRITC) and used as an oligonucleotide probe in *in situ* hybridization experiment too. The red fluorescent signal of the probe was detected with epifluorescent microscopy. A universal bacterial oligonucleotide probe EUB 338 (Amman *et al*., 1995) also labelled with TRITC, was used as a comparative probe. The *Salmonella* cells were clearly identified in both cases, intensity of the signal being comparable for both probes.

Due to high specificity and simplicity of the DNA isolation and PCR procedures, the described method could be used as a confirmation method included in Salmonella detection instead of biochemical and serological tests. Since certain components of foods can inhibit polymerase chain reaction, it is reasonable to use a preceding clean-up procedure, such as immunomagnetic separation, for isolation and concentration of *Salmonella* cells from food samples. Since there is a demand of detecting one single cell in 25 grams of the food sample, it is consequently required to include pre-enrichment or enrichment steps lasting a certain period of time. With the introduction of immunomagnetic separation and polymerase chain reaction, the entire procedure can, however, be significantly shortened.

POVZETEK

Bakterije rodu *Salmonella* so patogene bakterije, ki se prenašajo z živili in lahko povzročajo zelo resna obolenja pri ljudeh in živalih. Pomanjkljivost uveljavljenega postopka za odkrivanje salmonel je predvsem njegova dolgotrajnost, saj vključuje predobogatitev, obogatitev, izolacijo čiste kulture in potrjevanje značilnih kolonij. V prispevku smo predstavili specifično metodo za odkrivanje salmonel, ki temelji na pomnoževanju dela gena za 16S rRNK v verižni reakciji s polimerazo (PCR). Lin in Tsen sta l. 1996 objavila metodo, temelječo na prepoznavanju dela gena za 16S rRNK. S predhodno analizo nukleotidnih zaporedij male ribosomske podenote salmonel smo ugotovili, da začetna oligonukleotida 16SF1 in 16SIII ne prepoznata vseh bakterij omenjenega rodu. Začetni oligonukleotid 16SF1 smo modificirali v bolj specifičen reverzni oligonukleotid in razvili nov, proti 3' koncu usmerjen začetni oligonukleotid. Specifičnost metode smo primerjali s specifičnostjo postopka, ki ga je razvil Aabo s sod. (1993), na 87 različnih sevih salmonel in 30 sevih bakterij družine *Enterobacteriaceae*. Začetna oligonukleotida 16SF1 in 16SIII nista prepoznala naslednjih serotipov salmonel: Heidelberg, Weltevreden ter Houten, prepoznala pa sta tri različne seve bakterije vrste *Enterobacter cloacae*. Modificirani oligonukleotidi pa so bili 100% specifični. Reverzni začetni oligonukleotid smo označili s fluorescentnim barvilom TRITC in ga uspešno uporabili kot oligonukleotidno sondo *v in situ* hibridizaciji, fluorescentni signal sonde smo opazovali z epifluorescentnim mikroskopom.

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