Journal of Microbiological Methods 113 (2015) 50–56

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

Specific amplification of bacterial DNA by optimized so-called universal bacterial primers in samples rich of plant DNA

Samart Dorn-In [a,b,*,1], Rupert Bassitta [a,1], Karin Schwaiger [b], Johann Bauer [a], Christina S. Hölzel [a,c]

[a] Chair of Animal Hygiene, WZW, TUM, Weihenstephaner Berg 3, 85354 Freising, Germany
[b] Chair of Food Safety, Faculty of Veterinary Medicine, LMU, Schönleutnerstr. 8, 85764 Oberschleißheim, Germany
[c] Chair of Milk Hygiene, Faculty of Veterinary Medicine, LMU, Schönleutnerstr. 8, 85764 Oberschleißheim, Germany

ARTICLE INFO

Article history:
Received 19 February 2015
Received in revised form 7 April 2015
Accepted 7 April 2015
Available online 9 April 2015

Keywords:
Primer
PCR
16S rRNA
Bacteria
Plant
Archaea

ABSTRACT

Universal primers targeting the bacterial 16S-rRNA-gene allow quantification of the total bacterial load in variable sample types by qPCR. However, many universal primer pairs also amplify DNA of plants or even of archaea and other eukaryotic cells. By using these primers, the total bacterial load might be misevaluated, whenever samples contain high amounts of non-target DNA. Thus, this study aimed to provide primer pairs which are suitable for quantification and identification of bacterial DNA in samples such as feed, spices and sample material from digesters. For 42 primers, mismatches to the sequence of chloroplasts and mitochondria of plants were evaluated. Six primer pairs were further analyzed with regard to the question whether they anneal to DNA of archaea, animal tissue and fungi. Subsequently they were tested with sample matrix such as plants, feed, feces, soil and environmental samples. To this purpose, the target DNA in the samples was quantified by qPCR. The PCR products of plant and feed samples were further processed for the Single Strand Conformation Polymorphism method followed by sequence analysis. The sequencing results revealed that primer pair 335F/769R amplified only bacterial DNA in samples such as plants and animal feed, in which the DNA of plants prevailed.

© 2015 Elsevier B.V. All rights reserved.

1. Introduction

Most prokaryotes, which comprise bacteria and archaea, have three ribosomal RNAs (rRNA): one strand being part of the small ribosomal subunit (16S rRNA) and two strands that are part of the large ribosomal subunit (5S and 23S rRNA). The 23S rRNA contains highly variable regions and was reported to be suitable for the identification of bacterial species. However, the 23S rRNA was rarely used for quantifying and identifying bacterial DNA (Gaibani et al., 2013; Hunt et al., 2006). The 16S rRNA-gene-sequence is present in almost all bacterial species, contains also hypervariable regions, and its function is highly conserved. Additionally, the sequence of the 16S rRNA-gene (1500 bp) is large enough for the purposes of informatics (Janda and Abbott, 2007). Therefore, the 16S rRNA-gene has been used in many studies to identify the genus or even species of bacteria as well as to quantify the amount of bacterial contamination in samples by quantitative Polymerase Chain Reaction (qPCR, Czekalski et al., 2012; Klindworth et al., 2012; Kramski et al., 2011; Singh et al., 2014; Vital et al., 2013; Yarza et al., 2014).

For the amplification of target DNA by PCR with the purpose of genus or species differentiation, primers are generally designed to bind to the conserved regions and amplify variable regions, so that sequence analysis allows the differentiation of PCR products (Schmalenberger et al., 2001). However, some parts of the conserved region of the 16S rRNA from bacteria are similar to the conserved region of the 18S rRNA of mitochondria of eukaryotes (plant, animal and fungi) and the 16S rRNA of archaea and of chloroplasts of plants (Hanshew et al., 2013). These circumstances challenge the design of so-called universal bacterial primers for the detection and quantification of the total amount of 16S rRNA-genes in samples such as food, feed, feces and environmental samples, which contain DNA of plants or archaea; problems might also be posed by DNA of fungi or even of animals. Many of the existing universal primers for bacteria bind to non-target DNA in environmental samples or samples of animal origin, resulting in the co-amplification of non-target sequences (Ding et al., 2013; Gaibani et al., 2013; Galkiewicz and Kellogg, 2008; Hanshew et al., 2013; Rastogi et al., 2010; Ziegler et al., 2013). Some studies successfully applied procedures to exclude amplified plant DNA from the PCR product by separating the DNA fragments by electrophoresis (Sakai et al., 2004; Sun et al., 2008). However, non-target PCR products can be excluded by electrophoresis only if the length of amplified DNA fragments from bacteria and plants is clearly different. Additionally, those primer pairs are unsuitable for qPCR, since non-target DNA will also be quantified.

* Corresponding author at: Chair of Food Safety, Faculty of Veterinary Medicine, LMU, Schönleutnerstr. 8, 85764 Oberschleißheim, Germany.
E-mail address: D.Samart@ls.vetmed.uni-muenchen.de (S. Dorn-In).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.mimet.2015.04.001
0167-7012/© 2015 Elsevier B.V. All rights reserved.

The aim of this study was to provide a list of primers/primer pairs, which are suitable for the quantification of bacterial DNA by qPCR in food, feed or environmental samples rich of DNA from plants, archaea, fungi, or animal tissues. As an additional request, the region which is amplified by those primer pairs should constitute a non-conserved region, so that the identification of bacterial species/genera in samples by sequence analysis remains possible.

2. Materials and methods

2.1. Control strains

Escherichia coli, isolated from a manure sample at the Chair of Animal Hygiene, WZW, TUM, Germany (Hölzel et al., 2010) was used to generate the standard curve for qPCR. In addition, eight bacterial strains were used in order to validate the polyacrylamide-gel-electrophoresis. Six of these strains were purchased from the German Collection of Microorganisms (DSM): *E. coli* (DSM 1103), *Streptococcus agalactiae* (DSM 2134), *Staphylococcus aureus* (DSM 2569), *Corynebacterium mycetoides* (DSM 20148), *Mycoplasma bovis* (DSM 22781), *Helcococcus ovis* (DSM 21504). Two field strains were isolated and identified at the Chair of Animal Hygiene by standard microbiological methods including biochemical tests (BBL Crystal, BD): *Trueperella pyogenes* and *Klebsiella pneumonia*.

2.2. Validation of the polyacrylamide-gel-electrophoresis

Since the PCR products of each primer pair differed in length and nucleotide sequence, different conditions of polyacrylamide gels (MDE®-concentration) and electrophoresis (running time) had to be validated and optimized for the PCR products of each tested primer pair. In total, there were 13 samples applied for this purpose: the six DSM-strains and two field strains mentioned above (see Section 2.1), and five samples: three samples of ruminal fluid from cows, one feed samples and one meat sample.

2.3. Tested samples

After the validation of the SSCP-method by using control samples, there were in total 13 samples used for testing the specificity of tested primer pairs: three plant samples (two ryegrasses: *Lolium* spp. and one corn seed: *Zea mays*), two samples of soil from grain fields, and one of each following sample type: animal feed for pigs, human feces, pig feces, pig manure, slurry from a pig farm, solid dung, ferments from a biogas digester, and ruminal fluid from a cow. Plant samples were collected from grassland. The components of animal feed were barley, wheat, oat, corn, soybean, alfalfa meal and molasses.

2.4. DNA extraction

The DNA extraction followed the instructions of a commercial kit ("PowerSoil Kit", MoBio, Germany). Maximum caution was exercised in order to avoid additional bacterial contamination when taking plant samples for DNA extraction; this included the use of gloves and sterile equipment. The internal content of the young corn seed and of the stem of ryegrass was used for DNA extraction. Five milligrams of plant content in 250 ml sterile water was processed for genomic DNA extraction. For the other samples: feces, manure, slurry, solid dung, feed, ferment from biogas digester, soil and ruminal fluid, 1 g of sample and 9 ml of normal saline (buffered pH 7.4) in a 15 ml centrifuge tube were vortexed at maximum speed for 1 min. Then, 250 μl of the sample suspension (dilution 10^{-1}) served as material for the DNA-extraction. DNA from each tested sample was extracted in duplicate. Both extracts were pooled and served as template for qPCR-analysis.

In order to prepare a standard for qPCR, 10 ml of pig manure (in a 15 ml Falcon® tube) was irradiated with 450 kGy Gamma rays (Synergy Health, Germany) to destroy the existing DNA, thus representing only manure matrix. The irradiated pig manure was artificially contaminated with *E. coli* (see Section 2.1). The total cells were harvested from overnight culture (in standard-nutrient broth No. 1, Merck, Darmstadt, Germany) by centrifugation (4694 ×*g*, 10 min), resulting in a concentration of approximately 4×10^{11} cfu ml^{-1} manure. Five aliquots of 250 μl of artificially contaminated pig manure were processed for DNA extraction, then all five extracted samples were pooled and serially diluted. The standard curve covered concentrations from 10^{11} to 10^{3} in tenfold dilution steps.

2.5. Primer analysis and primer selection

Table 1 shows a list of 42 sequences of primers specific for bacteria, analyzed for their mismatches of base pairs with sequence of chloroplast and mitochondria of plant as well as their specificity to the 16S rRNA-gene of bacteria. 38 sequences were adopted from other studies and four sequences were designed/modified for this study, namely primer 335F, 769F, 769R and 1519R. The forward primer 335F was modified from primer 338F (Bakke et al., 2011): Three bases were added at 5′ end, this added one more mismatch to mitochondria of plant, but at the same time still bound to most bacteria. A forward primer 769F and its complementary sequence, a reverse primer 769R, were newly designed. Both primers had four to five mismatches to chloroplast and two mismatches to mitochondria. Primer R1519 was modified from primer 1522R (Lu et al., 2003) and Amp-R (Wang et al., 2002) to increase mismatches to chloroplasts as well as to mitochondria of plants. The forward primer 769F, its complementary reverse primer 769R, the forward primer 335F and the reverse primer 1519R were based on the 16S rRNA sequence of aerobic and anaerobic bacteria which were found in food, feces, anaerobic environment such as rumen or biogas digester. In total, 80 sequences of 16S rRNA of aerobic and anaerobic bacteria from GenBank (NCBI) were analyzed for the design of these four primers. The sequences of mitochondria and chloroplasts for the alignment (mismatch analysis) with the sequence of the listed primers were also taken from GenBank (NCBI, Table 2).

After analyzing the mismatch of primers to the sequence of chloroplast and of mitochondria of plants, eight primers (primer 335F, Com1, Com2, 769F, 769R, 799F, 783R and 1519R) were selected for further mismatch analysis with 18S rRNA-gene of mitochondria of animals and fungi and 16S rRNA-genes of archaea. Table 2 shows a summary of mismatch analysis of eight selected primers with DNA of plants, archaea, animal tissue and fungi.

Resulting from this analysis, the combination of primer 335F and 769R should not bind to the rRNA-gene of plant, archaea, animal tissue and fungi. The combination of primer Com1 and 769R might possibly amplify some species of archaea in group 3 and mitochondrial DNA of plant and of fungi in genus of *Penicillium* (group 3, Table 2). Primer 1519R had at least five mismatches to rRNA of plant, fungi, animal tissue and most species of archaea (except some species such as *Methanosphaera stadtmanae* and *Thermococcus gammatolerans*, which contain one and three mismatches, respectively).

Reasonable combination of these eight primers resulted in six primer pairs (Table 3) which were further tested with 13 samples. The reverse primers from Table 3 were phosphorylated at the 5′ end, which enables the separation of their PCR products by the SSCP method for subsequent sequence analysis. Primer pair No. 1 (Com1/Com2) was used as a reference primer pair, since it can amplify DNA from most bacteria and probably from plants (see mismatch analysis, Table 2), at the same time being suitable for PCR–SSCP, since it amplifies a non-conserved region.

2.6. Quantitative PCR (qPCR)

The qPCR assay was performed using a LightCycler® 480 Instrument II (Roche, USA). Each reaction contained a 20 μl mixture that included

Download English Version:

https://daneshyari.com/en/article/8421703

Download Persian Version:

https://daneshyari.com/article/8421703

Daneshyari.com