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DEVELOPMENT OF cPCR TECHNIQUE FOR DETECTION AND ENUMERATION OF *Prevotella bryantii*

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ABSTRACT

Competitive PCR (cPCR) system for the detection and enumeration of *Prevotella bryantii* cells in rumen samples was developed. PBB_14 primer, specific for *P. bryantii* was used as the reverse PCR primer and EUB 338 bacterial primer as the forward primer. An internal DNA control, containing primer specific sequences at 5' and 3' ends, but lacking 41 bp at the middle of the sequence, was constructed. C-PCR products were quantified by capillary electrophoresis and the results were calculated with regression equation (Reilly and Attwood, 1998). By coamplification of *P.bryantii* B_14 genomic DNA extracted from known numbers of cells and internal control, standard curve was constructed which enables quantification of *P.bryantii* cells in the range of 2.15×10^3 to 4.3×10^4 cells.

Key words: microbiology / bacteria / Gram-negative bacteria / *Prevotella bryantii* / rumen / analytical chemistry / cPCR / capillary electrophoresis

PRIPRAVA METODE cPCR ZA HITRO ODKRIVANJE IN ŠTEVILČNO VREDNOTENJE CELIC BAKTERIJE *Prevotella bryantii*

IZVLEČEK

Razvili smo metodo kompetitivne PCR za odkrivanje in številčno vrednotenje bakterijskih celic *Prevotella bryantii* v vzorcih vampnega soka. Za namnoževanje gena 16S rRNK vrste *P. bryantii* smo uporabili univerzalni bakterijski začetni oligonulkleotid EUB 338 ter za to vrsto specifičen začetni oligonukleotid PBB_14. Pripravili smo interno kontrolo, ki je imela isti prepoznavni mesti za začetna oligonukleotida na 5' in 3'. Od tarčne DNA, tj. genomske DNK vrste *P.bryantii* seva B_14, se je razlikovala le po deleciji 41 bp na sredini nukleotidnega zaporedja. Namnožene produkte cPCR smo ovrednotili s kapilarno elektroforezo in rezultate izračunali z regresijsko enačbo (Reilly in Attwood, 1998). Na osnovi sočasnega pomnoževanja tarčne DNK, ki smo jo izolirali iz znanega števila celic čiste kulture *P.bryantii* seva B_14 in interne kontrole, smo izdelali umeritveno premico, ki omogoča številčno vrednotenje bakterijskih celic *P.bryantii* v območju $2{,}15 \times 10^3$ do $4{,}3 \times 10^4$.

Ključne besede: mikrobiologija / bakterije / gram negativne bakterije / *Prevotella bryantii* / vamp / analitska kemija / cPCR / kapilarna elektroforeza

INTRODUCTION

Microorganisms are integral components of most ecosystems on our planet. They are essential for global cycling of nutrients and they commonly affect the health of higher organisms in a positive or negative way. Understanding the role of microorganisms in nature requires

knowledge of their specific population dynamics and of interactions between particular microbes and other organisms. Studying the role of specific microorganisms in nature remains difficult mainly because of complexity and diversity of natural microbial populations, improper isolation techniques and insatisfactory culturability in *in vitro* conditions (Amann *et al.*, 1995). Therefore the detection and enumeration of many groups of microbes are limited to the detection and enumeration of their DNAs (Lee *et al.*, 1996). *In situ* 16S rRNA probing has been successfully used for localisation of specific bacteria in number of environments including plant roots (Ludwig *et al.*, 1997), biofilms (Neef *et al.*, 1996), aquatic ecosystem (Siering and Ghiorise, 1997) and the rumen (Amann *et al.*, 1990, Tepšič in Avguštin, 1997). This approach doesn't allow the detection of species in low abundances (Amann *et al.*, 1995) however.

The sensitivity and specificity performances of PCR make it the best available method for the purpose of detecting nucleic acids present in low amounts in biological samples. Because of the low tube to tube reproducibility of PCR amplification, it is difficult or even imposible to use conventional PCR technique in a quantitative manner. Presently, the most efficient, reproducible, and flexible method for molecular quantification is competitive PCR (cPCR). The technique is based on co-amplification of two similar template species, the wild-type sequence which is to be quantified, and reference template known as internal control, which have similar length and share the same primer recognition sites. During the amplification, two similar templates compete for the reagents such as enzymes, Mg^{++}, deoxynucleoside triphosphate (dNTP) and the same primer set and consequently, amplify at the same rate independently of the number of cycles and any predicable or unpredicable variable influencing PCR amplification (Clementi *et al.*, 1995). All this should lead to reliable quantification. Therefore the starting mass of the target DNA can be approximated by comparing the ratio of the final mass of the competitor to its initial mass with the final mass of the target sequence. (Lee *et al.*, 1996).

Prevotella spp. are members of the bacterial phylogenetic group *cytophaga-flavobacter-bacteroides* (CFB) (Woese, 1987). The CFB bacteria are frequently isolated from many natural and man-made ecosystems. By their ability to degrade macromolecules such as celullose, and other plant structural polysaccharides, they are of considerable practical importance. *Prevotella* spp. are recognised as one of the most numerous strictly anaerobic Gram-negative bacteria inhabitating the rumen. *Prevotella bryantii* $B_1$4 is a type strain of noncelluloytic species that actively degrades xylan (Avguštin *et al.*, 1997, Marinšek Logar, 1999). Because of its presumably important role in the rumen ecosystem a rapid and sensitive detection system would be very useful. We developed a cPCR system to quantify this organism in the rumen samples and to estimate its distribution and dynamics contribution.

MATERIALS AND METHODS

Bacterial strains

Strain *Prevotella bryantii* ($B_1$4, Russel, 1983; DSM 11371) was cultivated anaerobically according to the Hungate's technique for cultivation of anaerobic microorganisms (Bryant, 1972) in 7.5 ml M2 broth (Hobson, 1969) at 37^o C.

DNA extraction

Genomic DNA of pure culture *P. bryantii* $B_1$4 was extracted according to the method described by Reilly and Attwood (1998) with some modifications. Bacterial cells were grown overnight (OD_{654}=1.66) and 1ml of culture volume was diluted with 5 % polyvinylalcohol (Sigma) (1:30) and counted by phase-contrast microscopy on a Petroff-Hausser counting chamber (Hausser Scientific Partnership Horsham, PA, 3900) according to the manufacturers

recomendations. The rest of the culture was centrifuged for 10 min at 10000×g and 4° C. The pelleted cells were resuspended in 1 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH=8) and added to 1.2 g of sterile zirconia-silica beads (Biospec products, Bartesville). Centrifugation at 12000×g for 10 min at 4° C followed. The pellet and beads were rinsed twice in saline-EDTA (0.15 M NaCl, 0.1 M EDTA) before final resuspension in 750 µl of saline EDTA. Physical disruption was performed with a Mini-beadbeater (Mini-beadbeater 3110 BX, Biospec products, Bartesville) at maximum speed for two intervals of 2 min each, with one minute incubation on ice between each treatment. Phenol-chloroform-isoamyl alcohol (25: 24: 1) was added and mixed with the sample, and the mixture was centrifuged at 12000×g for 15 min 4° C. The aqueous phase was removed, and the interface was reextracted with TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH=8). The combined aqueous phases were repeatedly extracted with the phenol-chloroform-isoamyl alcohol until the proteins remained at the interface. Nucleic acid was precipitated with isopropanol and centrifuged at 10000×g for 20 min at 4° C. Pellet was washed in 1 ml of 70 % ice cold ethanol and then centrifuged at 10000×g for 20 min at 4° C. The air-dried DNA pellet was resuspended in 50 µl TE buffer pH=7.4 and was stored at –20° C until required.

PCR primers and amplification

The primer used for amplification of 16S rRNA genes were as follows: universal bacterial forward primer EUB338 (Amann *et al.*, 1990; 5' gctgcctcccgtaggagt'3) labelled with 6-carboxy-4'5,5'-dichloro-2',7'-dimethoxyfluorescein (JOE) at 5' end and *P.bryantii* PBB_14 reverse primer (Avguštin *et al.*, 1994; 5' cgcttcctgtgcactcaagt '3). The PCR mixtures contained 10x *Taq* buffer II, (Gibco BRL) 0.2 mM each dATP, dTTP, dCTP and dGTP (Gibco BRL), 1.25 mM $MgCl_2$ (Gibco BRL), 2 U *Taq* DNA polymerase (Gibco BRL) and 10 pM of each primer. The PCRs were performed in final volume of 20 µl and thermocycling was carried out in a thermo cycler Gene Amp PCR System 2400 (Perkin Elmer). The PCR amplification conditions were as follows: denturation at 95° C for 5 min, 25 cycles of amplification (30 s at 95° C, 30 s at 52° C, 40 s at 72° C), with final cycle at 72° C for 7 min. PCR products were separated by agarose electrophoresis, stained with ethidium bromide, and visualised by UV transillumination.

Competitive PCR (cPCR)

The cPCR amplification conditions and reaction mixtures were the same as described for PCR. The concentratios of target genomic DNA and of internal control were changing only.

Quantification of PCR products

PCR products labelled with fluorochrome JOE were quantificated by capillary electrophoresis ABI Prism 310 Genetic Analyzer (Perkin Elmer). Before analysis PCR products were (according to the fluorescence intensity of fragments in agarose gels) properly diluted. One µl of diluted PCR product was then added to 12 µl of deionised formamide (Gibco BRL) and 0.5 µl of size standard TAMRA 350 bp (Perkin Elmer) and mixed. The mixture was denaturated for 3 min at 95° C and chilled on ice before being loaded onto the capillary electrophoresis.The experimental data were analysed by GeneScan^{TM} Anaysis software 2.1 and respresented in electrophoreogram.

RESULTS AND DISCUSSION

Following the successful detection of bacterial cells of *P.bryantii* in the rumen samples with fluorescent 16S rRNA targeted oligonucleotide probes (Tepšič and Avguštin, 1997), the logical

step seemed to be the quantification of *in situ* hybridized microbial cells observed with epifluorescent microscope. However, such quantification proved to be too demanding without computer assisted digital image analysis system as the method for routine examinations of complex microbial samples (Tepšič, 1999). Therefore we developed a cPCR method for the enumeration of *P.bryantii* bacterial cells in the rumen fluid samples. The addition of internal control in PCR reaction controled the variation among reactions and allowed the quantification of PCR products. cPCR technique was originally developed for quantification of HIV virus type 1 3B and has been later extended to bacterial quantification in the environment (Leser *et al.*, 1995, Lee *et al.*, 1996, Reilly and Attwood, 1998).

Construction of internal control

The construction of the internal DNA control was based on the analysis (WebCutter, Internet address) of the 16S rDNA sequence of the *P.bryantii* B_14 strain (Accession No. AJ006457). The 327 bp PCR product, amplified with PBB_14 and EUB 338 primers, was digested with *AvaII* restriction endonuclease (Gibco BRL) to give three fragments of 158, 41 and 128 bp. After the separation with agarose gel electrophoresis the larger fragments (158 and 128 bp) were purificated from the gel and ligated with T4 DNA ligase (Gibco BRL). Two µl of the ligation reaction was then used in the subsequent PCR and the products were separated again in the agarose gel. The 286 bp fragment was purified from the gel and used as an internal control for the cPCRs (Figures 1 and 2).

Internal control (286 bp) differes from the template sequence only in length (12.5 %). It kept the same recognition sites for the primers as the tamplate DNA.

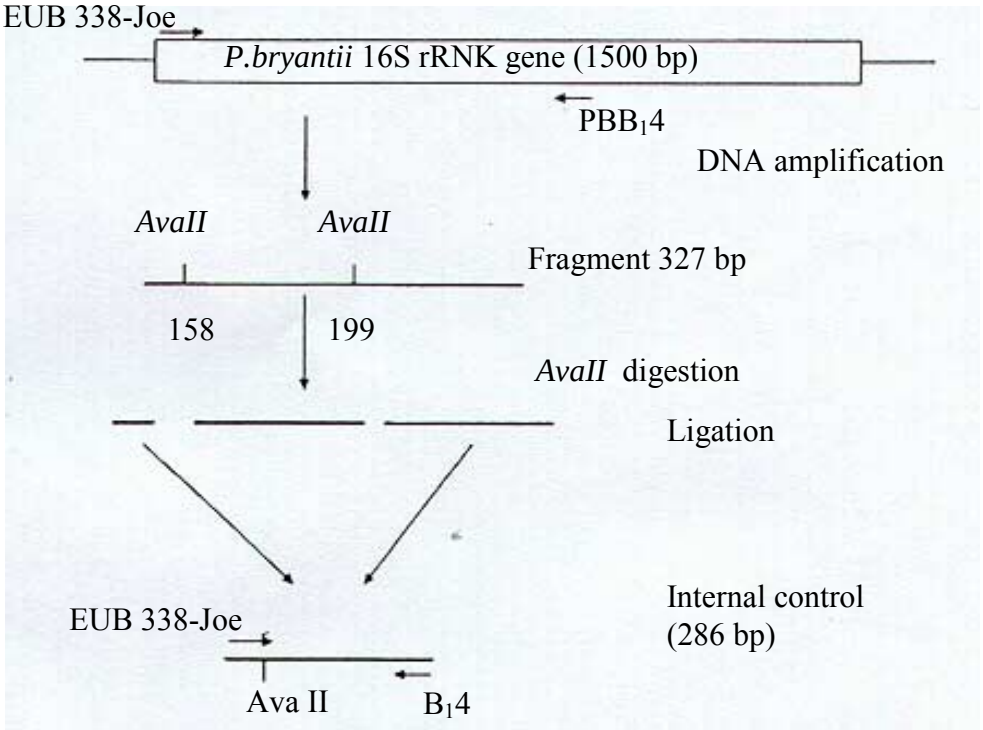


Figure 1. Construction of internal control with restriction endonuclease *AvaII*.
Slika 1. Shema priprave interne kontrole z restrikcijskim encimom *AvaII*.

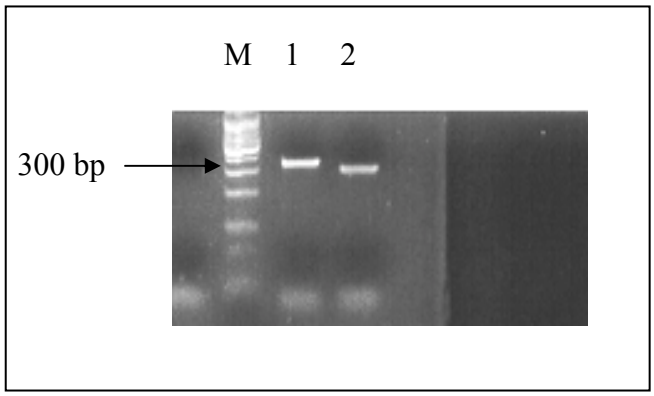


Figure 2. Lane 1= PCR product from *P.bryantii* $B_1$4 genomic DNA; lane 2= internal control; M=100 bp standard (Promega).
Slika 2. Linija 1= PCR pomnožek genomske DNK seva *P.bryantii* $B_1$4; linija 2= interna kontrola; M= 100 bp standard (Promega).

The efficiency of internal control amplification.

Since cPCR is the most accurate when the target (i.e. genomic DNA from strain *P.bryantii* $B_1$4) and internal control are coamplified in equimolar proportions (Reilly and Attwood, 1998), it was necessary to determine the optimal internal control concentration. The relative amplification efficiencies of target and internal control DNA were determined from a plot of the ratio of log target peak area to internal control peak area against the log concentration of internal control DNA (Reilly and Attwood, 1998). Coamplification of DNA from 4.3 x 10^3 *P. bryantii* $B_1$4 cells (10^{-4} diluted genomic DNA) with dilutions of internal control (10^{-4} to 10^{-6}) (Figure 3, Table 1) resulted in a line with a slope of 0.98 and regression of 1.0 (Figure 4). This indicates equivalent amplification efficiencies of the target and control DNAs. The line intersects the x axis at –5, indicating that the optimal dilution of the internal control for detection of 4.3 x 10^3 *P. bryantii* $B_1$4 cells is 10^{-5}.

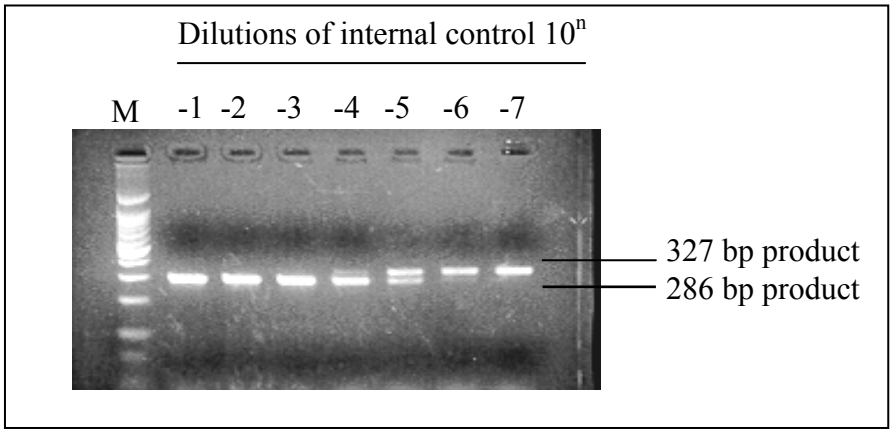


Figure 3. cPCR products. Line 1 to line 7 = coamplified genomic DNA from strain *P.bryantii* $B_1$4 (10^{-4}) with serial diluted internal control (10^{-1} - 10^{-7}); M=100 bp standard (Promega).
Slika 3. Elektroforetska ločitev produktov c-PCR, v kateri smo pomnoževali serijsko redčene interne kontrole (10^{-1} - 10^{-7}) in 10^{-4} redčeno genomsko DNK. M = velikostni standard (Promega, 100 bp).

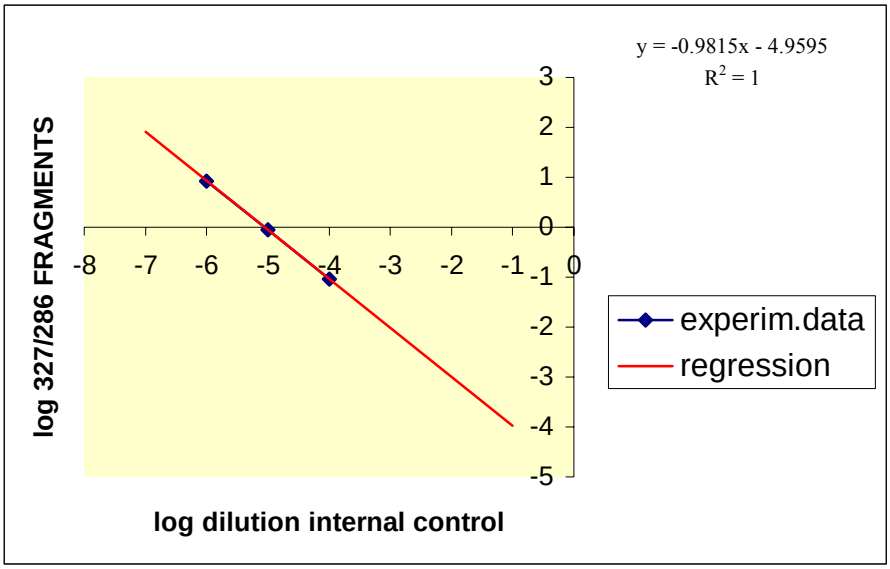


Figure 4. Standard curve construction for determination of optimal dilution of internal control.
Slika 4. Umeritvena premica za določitev optimalne redčitve interne kontrole.

Construction of standard curve for quantification of *P. bryantii* $B_1$4 cells in biological samples.

Optimal dilution of internal control was used to construct a standard curve by coamplification with *P. bryantii* $B_1$4 DNA extracted from known number of cells. DNA extracted from 3.3 x 10^9 *P. bryantii* $B_1$4 cells ml^{-1} was first serially diluted (10^{-3} – 10^{-7}) and then coamplified with the 10^{-5} dilution of the internal control. A plot of the ratio of log peak areas from both of the targets (Figure 5) against the log of *P. bryantii* $B_1$4 cell equivalents (based on the amount of DNA extracted per cell; Table 2) resulted in a line with a slope of 1.29 and regression of 0.87 (Figure 6). These results show that DNA extracted from 2.15×10^3 to 4.3×10^4 cells gives a linear response and could be used for quantification of *P. bryantii* cells within this range. For a wider range quantification of *P. bryantii* cells several standard curves should be constructed however, and this is what we are at present working on.

Table 1. Peak aeras of c-PCR products of target DNA and internal control after the analysis on capillary electophoresis ABI Prism 310
Preglednica 1. Površina vrhov cPCR produktov tarčne DNK in interne kontrole po številčnem ovrednotenju s kapilarno elektroforezo ABI Prism 310

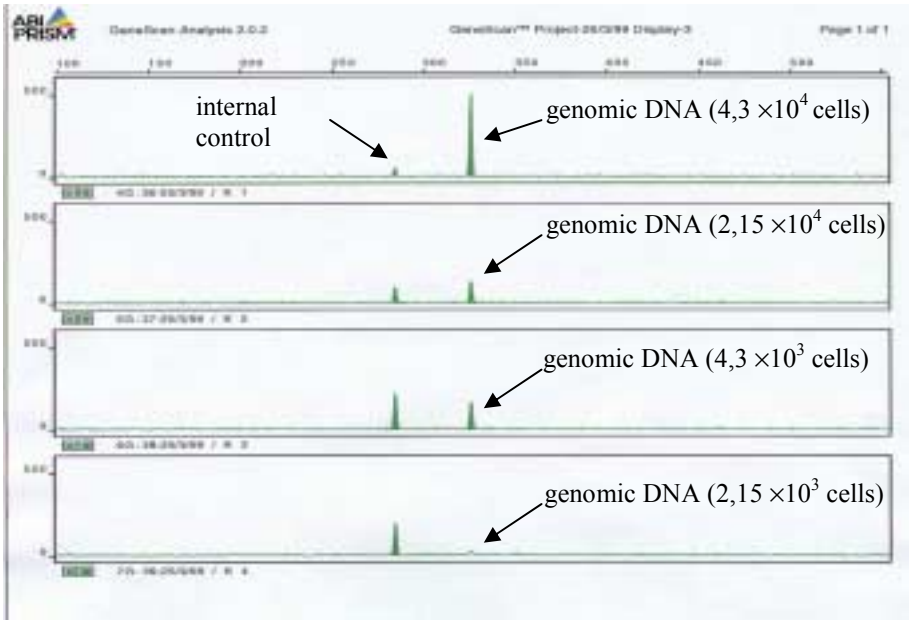
	Peak Areas / Površine vrhov	
• Dilutions of internal control • Redčitve interne kontrole	* target DNA (327 bp) * tarčna DNK (327 bp)	internal control (286 bp) interna kontrola (286 bp)
10^{-4}	759	8957
10^{-5}	4126	4599
10^{-6}	4049	480

- dilution of target DNA was 10^{-4} (4.3×10^4 *P. bryantii* sev $B_1$4 cells)
- redčitev genomske DNK je bila 10^{-4} (4.3×10^4 celic *P. bryantii* sev $B_1$4)

* at dilutions 10^{-1} to 10^{-3} and 10^{-7} of the internal control used only one of the targets was amplified
* pri redčitvah 10^{-1} do 10^{-3} in 10^{-7} se je pomnoževala samo ena od tarč

Table 2. Cell equivalents of dilutions of genomic DNA (*P. bryantii* $B_1 4$ DNA)
Preglednica 2. Celični ekvivalenti redčitev genomske DNK

Dilutions of genomic DNA Redčitev genomske DNK	Cell equivalents Celični ekvivalenti	Dilutions of genomic DNA Redčitev genomske DNK	Cell equivalents Celični ekvivalenti
10^{-3}	4.3×10^5	$10^{-5.5}$	2.15×10^3
10^{-4}	4.3×10^4	10^{-6}	4.3×10^2
$10^{-4.5}$	2.15×10^4	10^{-7}	4.3×10^1
10^{-5}	4.3×10^3		



Dye / Sample peak		Minutes	Size	Peak Height	Peak Area
1G	1	17′ 24"	284.69	59	472
1G	2	18' 25"	325.76	503	4125
2G	3	17' 21"	284.46	102	902
2G	4	18' 22"	325.76	133	1264
3G	5	17' 18"	284.57	225	2085
3G	6	18' 18"	325.69	165	1518
4G	7	17' 13"	284.52	207	1874
4G	8	18' 19"	325.75	20	157

Figure 5. Electrophoreogram of cPCR products. Serial diluted target DNA (10^{-4}- $10^{-5.5}$) was coamplified with optimal dilution of internal control (10^{-5}).
Slika 5. Elektroferogram produktov cPCR, v kateri smo pomnoževali serijsko redčene tarčne DNK (10^{-4}- $10^{-5.5}$) in 10^{-5} redčeno interno kontrolo.

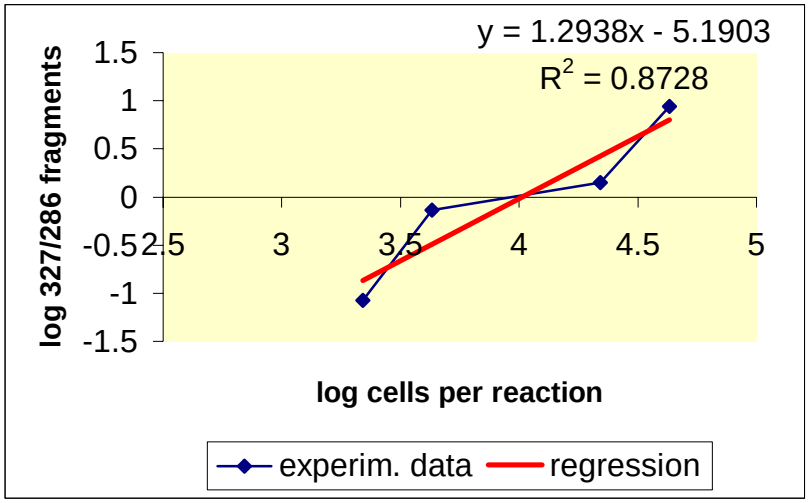


Figure 6. Standard curve for quantitation of *P. bryantii* $B_1$4 cells in rumen fluid samples.
Slika 6. Umeritvena premica za odčitavanje števila bakterijskih celic *P.bryantii* v vzorcih vampnega soka.

SUMMARY

Competitive PCR (cPCR) for detection and enumeration of *Prevotella bryantii* $B_1$4 cells in rumen fluid samples was developed. For amplification of the 16S rRNA genes universal forward primer EUB 338 and *P. bryantii* specific reverse primer $PBB_1$4 were used. An internal DNA control, based on the analysis of the 16S rDNA sequence of the *P.bryantii* $B_1$4 strain, was constructed. The 327 bp PCR product from *P.bryantii* $B_1$4 DNA contains two *AvaII* sites. After the digestion with *AvaII* restriction endonuclease the larger fragments (158 bp and 128 bp) were ligated and subsequently PCR amplified. The internal standard preserved the same recognition sites for the primers and differed from the wild type sequence only in short delection (41 bp) in the center of the sequence. Optimal dilution of internal control, which was amplified with the same efficiency as genomic DNA, was determined by coamplification of a single dilution of *P.bryantii* $B_1$4 DNA with serial dilutions of internal control. C-PCR products were quantified by capillary electrophoresis and the results were calculated using the regression equation. (Reilly and Attwood, 1998). Ratio of log target peak area to internal control peak area against to the log concentration of internal control DNA resulted in a straight line with a slope of 0.98, which indicated nearly equivalent amplification efficiencies of the target and control DNAs. That means that the ratio of final mass of the competitors depended only on ratio of their initial mass. Standard curve was constructed by coamplification of optimal dilution of internal control (10^{-5}) with *P. bryantii* $B_1$4 DNA extracted from known number of cells. Results showed that DNA extracted from 2.15×10^3 to 4.3×10^4 cells (dilutions 10^{-4}, $10^{-4.5}$, 10^{-5} and $10^{-5.5}$) gave a linear response and could be used for quantification of *P.bryantii* cells within this range.

POVZETEK

Razvili smo metodo kompetitivne PCR za odkrivanje in številčno vrednotenje bakterije *Prevotella bryantii* v vzorcih vampnega soka. Kot začetna oligonukleotida, ki omogočata pomnoževanje interne kontrole in genomske DNK seva *P. bryantii* $B_1$4, smo uporabili $PBB_1$4 kot proti 5' koncu obrnjeni ("reverse") oligonukleotid in EUB338 kot proti 3' koncu obrnjeni oligonukleotid (»forward«). $PBB_1$4 je zagotavljal specifičnost reakcije, saj prepozna le bakterije

vrste *P. bryantii* (Avguštin in sod., 1994). Interno kontrolo, ki je ključni dejavnik c-PCR, smo pripravili na osnovi že znane sekvence gena za 16S rRNK tipskega seva vrste *P.bryantii*. Po restrikciji z encimom *AvaII*, ki je rezal sekvenco dolgo, 327 bp na mestih 158 in 199, smo odstranili srednji del sekvence, bočna konca pa zlepili. Dobili smo interno kontrolo, ki je ohranila prepoznavni mesti za začetna oligonukleotida in se je od sekvence tarčne DNK razlikovala le po deleciji 41 bp. Optimalno redčitev interne kontrole, ki se je pomnoževala z enako učinkovitostjo kot tarčna DNK (genomska DNK seva *P. bryantii* $B_1$4), smo ugotovili s primerjavo serije reakcij, v kateri smo pomnoževali določeno količino genomske DNK z različnimi količinami interne kontrole. Namnožene produkte c-PCR smo vrednotili s kapilarno elektroforezo in rezultate izračunali z regresijsko enačbo (Reilly in Attwood, 1998). Logaritemsko razmerje površin vrhov tarčne DNK in interne kontrole ter logaritem serijskih redčitev interne kontrole (vrednosti y) so v regresijski enačbi dale premico z naklonom – 0,98, kar nakazuje skorajda popolnoma enakovredno pomnoževanje obeh tarč. To pomeni, da je razmerje količin nastalih končnih produktov izključno odvisno od razmerij količin tarčne DNK in interne kontrole na začetku reakcije. Umeritveno premico smo izdelali na osnovi pomnoževanja 10^{-5} redčene interne kontrole ter različnih količin tarčne DNK (genomske DNK seva *P. bryantii* $B_1$4), ki smo jo izolirali iz čiste kulture z znanim številom celic. Redčitve 10^{-4}, $10^{-4,5}$, 10^{-5} in $10^{-5,5}$ tarčne DNK so omogočile namnoževanje obeh produktov, kar omogoča s tako redčeno interno kontrolo številčno vrednotenje v območju $2{,}15 \times 10^3$ do $4{,}3 \times 10^4$ celic.

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