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DNA ISOLATION FROM EQUINE FAECAL EXCREMENTS

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ABSTRACT

Sampling and extraction procedures were elaborated for DNA isolation suitable for subsequent PCR amplification and other molecular manipulation experiments from horse excrements. Eleven Lipizzaner horses were split into two age groups and faecal excrements were collected as soon as possible after defecation and the DNA was isolated immediately after arrival to the laboratory. The excrements collected from the group of younger horses yielded on average 3-4 fold higher amounts of DNA than from the group of older horses. The variability of the DNA yield isolated on successive days from the same horse remained within 15% of the average yield. The DNA yield isolated on successive days from the same faecal excrement increased gradually from 4.61 to 7.96 μg g^{-1} faeces until 48 hrs after defecation and dropped only slightly to 7.51 μg g^{-1} after further four days. Our results indicate, that substantial microbial growth is taking place in the faecal excrements at least until two days after defecation. It is of utmost importance therefore, that the sampling is done as soon as possible after defecation if microbial community structure analysis of the equine colon and faeces is to be performed.

Key words: horses / molecular genetics / DNA / excrements / isolation / yield

OSAMITEV DNK IZ KONJSKIH IZTREBKOV

IZVLEČEK

Preučili smo postopke vzorčenja in osamitve DNK iz konjskih iztrebkov za potrebe nadaljnjih molekulsko bioloških poskusov kot npr. namnoževanje genov z reakcijo PCR. Enajst poskusnih konjev pasme lipicanec smo razdelili v dve starostni skupini in zbirali njihove iztrebke v čim krajšem času po iztrebljanju. Iz vzorcev smo osamili DNK takoj po prihodu v laboratorij. Iz vzorcev iztrebkov iz skupine mlajših konjev smo v povprečju osamili 3-4-krat več DNK kot iz vzorcev iz skupine starejših konjev. Variabilnost izplena DNK iz vzorcev iztrebkov istega konja, ki so bili odvzeti v zaporednih dneh, je odstopala le za 15% od poprečnega izplena. Izplen DNK, ki je bila osamljena iz istega vzorca v zaporednih dneh, se je povečal od 4,61 na 7,96 μg g^{-1} iztrebkov 48 ur po iztrebljanju in se je potem le rahlo znižal na 7,51 μg g^{-1} v naslednjih štirih dneh. Rezultati nakazujejo intenzivno mikrobno rast v fekalnih iztrebkih vsaj v prvih dneh po iztrebljanju. Zato je nadvse pomembno pravilno in čim hitrejše vzorčenje po iztrebljanju, če nameravamo preučevati strukturo mikrobne populacije debelega črevesa in iztrebkov pri konjih.

Ključne besede: konji / molekularna genetika / DNK / iztrebki / osamitev / izplen

INTRODUCTION

Microorganisms are essential inhabitants of the normal and healthy animal gastrointestinal tract. In animals feeding mainly on plant material i.e. ruminants but also other large and small herbivores, an intimate and obligatory symbiotic relationship has developed between hosts and gut microorganisms throughout evolution. The microorganisms are responsible for the breakdown of the main substrates like cellulose and hemicelluloses (Hobson and Stewart, 1997). The microbial community inhabiting the rumen is fairly well studied and understood, however, much less information is available for other herbivores.

The gastrointestinal tract of horses is generally characterised by a very large cecal and colonic capacity, which occupies approximately 45 and 15% of the total gut volume (Stewart, 1997). The actual capacities of certain parts of the digestive tract will obviously vary greatly according to the breed of the particular horse. The enlarged hindgut, comprising the large and small colon and the cecum, extends the residence time of the digesta particles which is somewhat similar to that in mature pigs (Stevens, 1977). The prolonged residence time makes possible the microbial degradation processes to occur and the organic polymers are degraded and subsequently transformed via fermentation to mainly volatile fatty acids and microbial biomass. Although a fair proportion of the available protein and carbohydrate is digested and absorbed prior to reaching the cecum, the volatile fatty acid production within the cecum alone accounts for about 30% of the digestible energy intake (Glinsky *et al.*, 1976).

Bacteria, fungi and protozoa were observed in equine gut. The most information seem to be available on protozoa, however, some authors have suggested that protozoa don't play an essential role in the fermentation of feedstufs in the equine hindgut (Moore and Dehority, 1993). The defaunation (removal of protozoa from the hindgut) had no significant effect on cellulose digestibility and the authors suggested that either protozoa are not very important in cellulose digestion or that the bacteria and fungi simply take over their role when protozoa are removed. Several anaerobic fungi, similar to the ruminal chytridiomycetal fungi from the family *Neocalimasticaceae*, were isolated and described from equine gut samples (Stewart, 1997). They are like their ruminal relatives active polysaccharide degraders but presumably play only a limited role in the equine gut due to a relative slow growth when compared to bacteria. Compared to what is known about ruminal bacteria, the knowledge about equine gut bacteria is very limited. From equine faeces and gut samples bacterial genera similar to ruminal were isolated i.e. streptococci, lactobacilli clostridia and *Bacteroides* (Smith and Crabb, 1961) and also lactate utilizers like *Veillonella* and bacteria similar to *Megasphaera* (Alexander and Davies, 1963). However, the small number of detailed studies that were done, showed, that although related to ruminal bacteria, equine gut isolates are phylogenetically different and probably represent new taxa (Lin and Stahl, 1995, Julliand *et al.*, 1999). Only a small number of studies applying molecular approaches for the studying of microbial community structure and dynamics was done to date and they were concentrated on establishing the predominant cellulolytic species and genera. *Fibrobacter* and *Ruminococcus* seem to be the prevalent fibre degraders in equine gut. Fibrobacter rRNA represented about 12% of total rRNA extracted from cecal sample of the pony (Lin and Stahl., 1995) whereas up to 10% of the total 16S rRNA was accounted to *R.flavefaciens* in cecal contents of ponies and donkeys (Julliand *et al.*, 1999). Nothing is known about other members of the bacterial community however.

Studying the community structure, its dynamics and the role of specific microbial species or genera in their natural habitats remains difficult mainly because of complexity and diversity of natural microbial populations, demanding and often improper isolation techniques and as a consequence insatisfactory culturability in *in vitro* conditions (Amann *et al.*, 1995). Molecular biology approaches have made possible such studies without isolation and cultivation, relaying on the detailed analysis of the specific genes, which are directly retrieved from the sample by

DNA isolation, PCR amplification, cloning and sequencing procedures (Amann *et al.*, 1995). The ruminal microbial community was recently investigated (Whitford *et al.*, 1998, Tajima *et al.*, 1999, Ramšak *et al.*, 2000) and clostridia and *Prevotella* were found to be the dominant bacterial genera inhabiting the rumen. In this paper we report of preliminary experiments preparing the initial stages for a molecular study of the microflora inhabiting the equine gastrointestinal tract, i.e. sampling and DNA isolation procedures.

MATERIAL AND METHODS

Horses included in the study

Eleven Lipizzaner horses were included in the study from Marc Lipizzaner breeding centre (Kranj, Slovenia). Horses were split into two age groups, first group comprising younger horses till four years of age , and the second group comprised horses older than four years (Table 1). The sampling of the faecal excrements was done from February till Aprill 2000 and the horses were kept outdoor during the day**.** The group of older horses was fed with hay ad libitum and with additional mixtures of oats and corn in the morning and oats, soya meal, sunflower grain and bran in the evening. Additional mineral vitamin/mixtures were supplied. The group of younger horses was fed on hay ad libitum as well with the addition of oats in the morning and in the evening and vitamin/mineral mixtures in the evening only.

Sampling of faecal excrements

Faecal excrements were collected immediately after the defecation, stored in a nylon bag and transferred immediately to the laboratory. The samples were stored at –4º C for a maximum of 10 minutes and the DNA isolation procedure was performed.

DNA isolation procedure

Total DNA was extracted by three different procedures. One was essentially the same as described by Tepšič and Avguštin (1999) with only minor modifications. The second procedure was like the one described by Ausubel and colleagues (1987) with modifications. Nine ml of PBS buffer (0.13 M NaCl, 0.07 M Na_2HPO_4 and 0.03 M NaH_2PO_4, pH 7.4) was added to 1 g of faecal sample and vigorously mixed on a vortex mixer for 2-3 minutes. The sample was than further macerated by a sterile glass rod and left on ice for sedimentation 30 minutes. Six mls of supernatant was collected and centrifuged at 12,000 x g for 10 min at 4º C. The supernatant was discarded and the pelleted cell were washed twice with ice cold PBS. The cells were than resuspended in 2 ml of PBS and split into two aliquots. Each aliquot was transferred to a sterile 2.0 ml microfuge tube, fitted with a screw cap and an o-ring washer. 1.2 g of sterile zirconium beads were added (0.6 g of beads with r = 0.5 mm and 0.6 g of beads with R = 0.1 mm) and the cells were then physically disrupted 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 12,000 × g for 10 min at 4° C. 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 12,000 × g for 5 min at 4° C. Pellet was washed in 1 ml of 70% ice cold ethanol and then centrifuged at 12,000 × g for 5 min at 4° C. The air-dried DNA pellet was resuspended in 40 µl TE buffer pH=8.0 and stored at –20° C until required. The third

procedure was essentially a mixture of the first two, the sample being treated first with PBS buffer as described in the second procedure. However, the sample was physically disrupted in saline EDTA as described by Tepšič and Avguštin (1997) and further treated according to the first procedure.

The quality of the isolated DNA was verified by agarose electrophoresis (1% TBE agarose gels, run at 5 V/cm for 30 – 45 min). The gels were analysed and documented by Bio-Rad GelDoc 1000 Documentation System.

DNA quantification

The DNA concentration was measured fluorometrically with DyNA Quant 200 apparatus as described earlier (Peterka *et al.*, 1997).

RESULTS AND DISCUSSION

Three different DNA isolation procedures were tried in order to find the most suitable and reliable method, which will also yield the highest quantities of the high molecular DNA. Although the third procedure yielded DNA with the highest molecular mass (Fig. 1), the second procedure was chosen for further work since it was the only procedure yielding DNA that was successfully used for further manipulations i.e. as the target for PCR amplification of ribosomal genes (not shown). This procedure was therefore used in the following experiments.

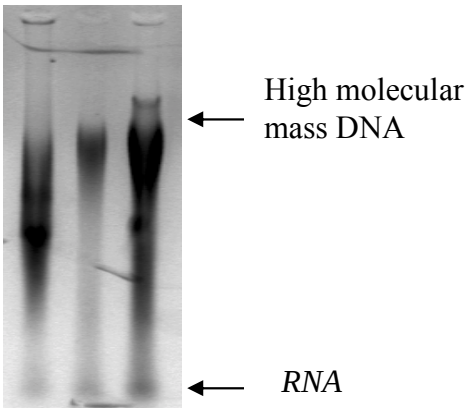

Figure 1. DNA isolated from the same sample of horse faecal excrements by three different procedures.
Slika 1. DNK osamljena iz istega vzorca konjskega iztrebka s tremi različnimi postopki.

Faecal excrements were collected immediately after the defecation from eleven horses (Table 1) and three parallel samples from each faeces were used for DNA isolation according to the second procedure. The DNA yield varied from 0.48 to 16.37 μg g^{-1} of faeces. The average yields were calculated for each sample from the three parallel treatments as well as standard deviations ($s=\sqrt{\frac{1}{n-1}\sum_{i=1}^{n}\left(X_i-\overline{X}\right)^2}$) (Table 1). The standard deviation calculations showed, that the variability of the DNA yield remained somewhere within 15% of the average yield for the sample (Table 1, Fig. 2). It soon became obvious that there are rather great differences between DNA yields from the faeces of younger and older horses. The average DNA yields from all samples from both groups of horses were therefore calculated and are presented in Fig. 3. The average DNA yield from the faeces of younger horses was 3 – 4 folds of the yield from the

faeces of the older horses and the difference was quite obviously exceeding the standard deviation range describing the variability as a consequence of isolation procedure. It seems reasonable to conclude that faeces of younger horses contains more DNA than of older horses, however we have no clear explanation for that phenomenon. It is known that coprophagy may appear in younger horses (Madigan *et al.*, 2000) and the microbial cells would therefore re-enter the gastrointestinal tract of the animal, however, most of them are probably degraded by acid and enzymatic lysis in the stomach. They could possibly stimulate faster growth of microorganisms in the hindgut and therefore higher quantities of the microbial DNA due to the protein and growth factors supply, which is released from their cells upon lysis in the stomach.

Table 1. DNA yield from faecal excrements of various horses
Tabela 1. Izplen osamljene DNK iz fekalnih iztrebkov različnih konjev

No. Št.	Name of the horse Ime in številka konja	Age group Starostna skupina	Average DNA yield Povprečni izplen DNK (µg DNA g^{-1} faeces)	Standard deviation Standardni odklon
1.	L988 Bonadea XXI 1988	o[a]	3.68	3.1981
2.	L942 Jadranka VI 1986	o	1.85	2.2143
3.	120 Bonadea 1996	o	3.75	1.8062
4.	137 Maestoso Jadranka 1997 [b]	o	5.05	3.5393
5.	L195 Neapolitano Jadranka VI 1996	o	5.64	1.0897
6.	138 Betalka 1997	y	9.88	4.4007
7.	188 Samira II 1998	y	11.78	4.9007
8.	214 Capra 1997	y	10.52	0.9681
9.	213 Pluto Samira 1997	y	10.91	3.1500
10.	214 Bonadea 1999	y	5.17	3.7493
11.	215 Jadranka 1999	y	8.97	3.5007

[a] – age groups: o = old, y = young (see material and methods).
[b] – The horse was treated as a member of the old group although born in 1997. It is stallion fed and treated as the older horses rather than the young.

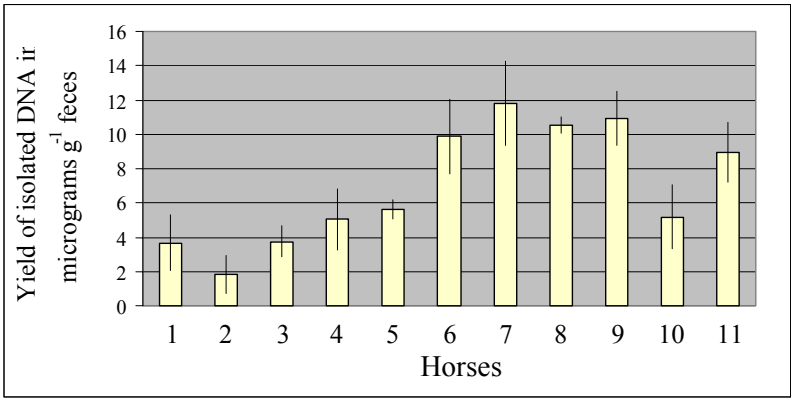

Fig. 2. DNA yield from faecal excrements of various horses. The average yields for the three parallel samples from each faeces and standard deviation values are shown.
Slika 2. Izplen DNK iz iztrebkov različnih konjev. Prikazani so povprečni izpleni iz treh vzporednih vzorcev vsakega iztrebka in standardni odkloni.

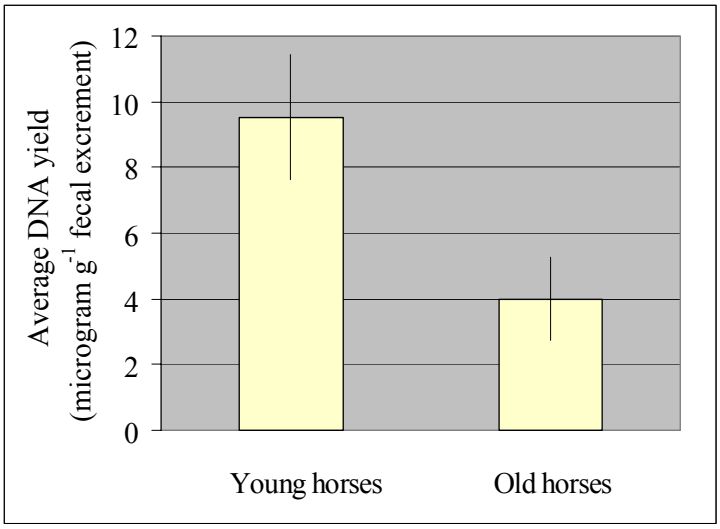

Figure 3. Average DNA yield from faecal excrements from groups of younger and older horses.
Slika 3. Povprečni DNK izplen iz iztrebkov pri mlajših in starejših konjih.

Due to the fairly large differences in DNA yields from different samples, faecal samples were obtained from one horse on consecutive days, in order to establish the variability range of the amount of DNA in faecal excrements of the same animal in different time. The faecal excrements were collected immediately after the defecation from the horse No. 1 (L988 Bonadea XXI 1988). The DNA yields are shown in Fig. 4.

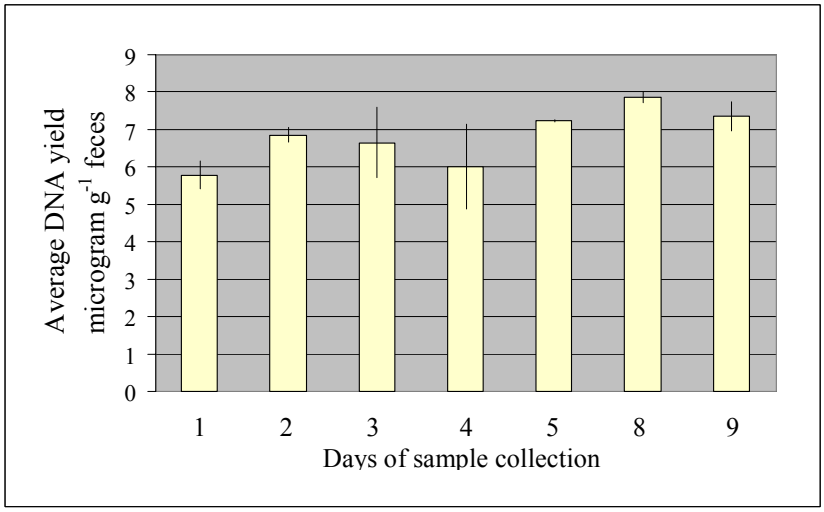

Fig. 4. Average DNA yield from faecal excrements of the same horse collected on successive days.
Slika 4. Povprečni izplen DNK iz vzorcev iztrebkov enega konja, odvzetih v zaporednih dnevih.

As it can be seen from Fig. 4., the variability of the DNA yield isolated from samples collected on successive days remained within approximately 15% of the average yield, which is acceptable given the complex isolation procedure with a high number of steps necessary in order to obtain DNA of suitable quality for further manipulations.

The influence of the incubation length of the sampled faecal excrements prior to the start of the isolation procedure and of the time spent between defecation and sampling was analysed too. The faecal sample was collected from the horse No. 8 (214 Capra 1997) on April 4th 2000 and stored in a perforated nylon bag on environmental temperature. Immediately before the isolation procedure was started, one gram of the sample was removed and the rest was reincubated on environmental temperature. The samples were processed immediately after arrival to the laboratory, and 3, 6, 24, 48 and 144 hrs after defecation. The results are presented in Table 2 and Fig. 5.

Table 2. Average DNA yield from one sample isolated during first six days after the collection of the sample.
Preglednica 2. Povprečni izplen DNK, izolirane iz enega vzorca v prvih šestih dnevih po vzorčenju

Time of isolation after the defecation (in hrs) Čas med defekacijo in začetkom postopka izolacije (v urah)	Average DNA yield Povprečni izplen DNK (μg g^{-1} faeces)	Standard deviation Standardni odklon
0*	4.61	1.539726
3	6.36	0.695601
6	6.76	0.855311
24	7.23	0.536020
48	7.96	0.647716
144	7.51	0.453984

* The time for the transport to the laboratory and the time spent in the laboratory before the actual start of the procedure must be taken into account.

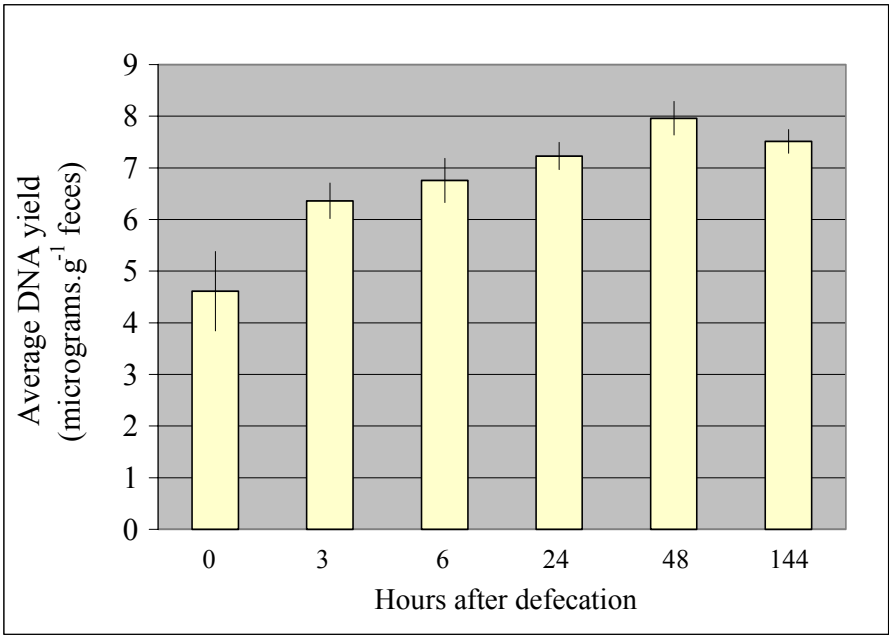

Figure 5. Average DNA yield from the same sample isolated during first six days after defecation.
Slika 5. Povprečni izplen DNK, izoliran iz enega vzorca v prvih šestih dnevih po iztrebljanju.

An obvious trend towards increasing of the DNA yield was observed with ageing of the faecal excrements. However, a slight decrease of the DNA yield after 144 hrs when compared to the value obtained after 48 hrs indicates that somewhere between day two and six the amount of the DNA in the faeces reaches the highest value and starts to decline after that. The most important are the first three hrs, however, since the average amount of the DNA increased for approximately 40% during that time. It is of utmost importance therefore, to isolate DNA as soon as possible after the defecation and to keep samples at temperatures, which will prevent further growth of microorganisms and changes in the structure of the population.

POVZETEK

V primerjavi s prežvekovalci vemo le malo o mikroorganizmih, ki naseljujejo konjski gastrointestinalni trakt. Bakterije, praživali in glive so nedvomno izjemno pomembni tudi v tem mikrobnem ekosistemu, saj zagotavljajo encimski potencial za razkroj rastlinske krme in so zato ključnega pomena pri energetskih pretvorbah. Doslej so raziskovalci osamili in opisali le nekaj bakterijskih vrst in rodov, katerih večina je bila podobna a ne povsem enaka vampnim bakterijam. Še vedno pa ni bil opravljen natančen pregled bakterijske populacije, ki naseljuje konjska prebavila, čeprav moderni molekulsko biološki pristopi že omogočajo takšne študije. V pričujočem prispevku opisujemo začetne preskuse, ki naj bi omogočili najboljše postopke vzorčenja in osamitve DNK, ključnih korakov vsake molekulsko biološke študije. Zbirali smo iztrebke enajstih konjev pasme lipicanec, ki smo jih razdelili v dve starostni skupini. Sprva smo osamili DNK s tremi različnimi postopki, vendar je le eden omogočil osamitev dovolj kakovostne DNK za nadaljnje molekulske preskuse. Iz enega grama iztrebkov smo pridobili od 0,48 to 16,37 µg DNK. Presenetljivo smo pridobili iz fekalnih vzorcev mlajših konjev kar tri do štirikrat več DNK na gram kot iz fekalnih vzorcev starejših konjev. Primerne razlage za opisani pojav še nismo našli. Izračuni standardnih odklonov kažejo, da je raznolikost izplena DNK iz treh vzporednih kontrol za vsak vzorec odstopala za največ 15% od povprečnega izplena DNK za vsak vzorec. Ugotovili smo tudi, da je raznolikost izplena DNK iz vzorcev iztrebkov enega konja, pridobljenih v zaporednih dnevih od prvega vzorčenja, ostala v okviru 15% od povprečnega izplena. To je po našem mnenju zadovoljivo glede na zahteven postopek osamitve DNK, z mnogimi vmesnimi koraki, ki so nujno potrebni, da pridobimo DNK primerne kakovosti za nadaljnje preskuse. Opazili smo tudi trend povečevanja količine DNK v vzorcih, ki smo jih starali. Zato je zelo pomembno, da vzorce iztrebkov zberemo in obdelamo v laboratoriju čim hitreje po iztrebljanju in jih v vmesnem času shranimo pri dovolj nizkih temperaturah, da preprečimo rast in s tem spremembe v strukturi mikrobne populacije.

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