Extraction of Microbial DNA from Rumen Contents Containing Plant Tannins

BioTechniques 31:294-298 (August 2001)

PCR amplifications are often used in molecular ecological studies (19,23), but these reactions can be inhibited by contaminants (7,20,24,25). One inhibitory compound is tannin, which is often present in protein-rich shrubs fed as a nitrogen supplement (16) to extensively grazing ruminants. Tannins inhibit the growth of many ruminal bacteria (8,14), and there is considerable interest in bacterial strains that are tolerant to tannins (16). To better understand the ecology of tannin-tolerant microorganisms, molecular ecological analyses may be conducted; however, there are no published protocols that specifically address the extraction of DNA from rumen samples rich in tannins.

In our protocol (Table 1), the key step is the removal of tannins using soluble polyethylene glycol (PEG; Table 1, step 4). The PEG binds to the tannins and other phenolics, which bind to the protein and cell debris upon lysis, forming a complex (13). When the lysate is centrifuged in the presence of phenol, the protein-tannin complexes accumulate at the interface between the organic and aqueous phases, resulting in a supernatant that contains DNA largely free of tannins. Hexadecyltrimethylammonium bromide (CTAB) and methanol are added to remove polysaccharides and soluble phenolics, respectively, but these steps were not specifically evaluated and were included only as precautionary measures.

Quebracho tannin was added in increasing amounts (0.5%, 1%, 2%, 4%, and 8%) to ground freeze-dried lucerne hay (100 mg), which is essentially free of condensed tannins. One milliliter of concentrated rumen fluid was mixed with the plant material, and microbial DNA was extracted from the preparation. A 16S rDNA PCR product could be successfully amplified from the DNA extracts using primers 27f (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492r (5′-TACGGYTACCTTGTTACGACTT-3′) (11). Each 20-µL PCR contained 5 µL 10× PCR buffer, 0.5 µL $MgCl_2$ (250 mM), 0.4 µL each dNTPs (10 mM), 10 pmol each primer, 1 U *Taq* DNA polymerase (Promega, Madison, WI, USA), and 0.5 µL DNA extract. Cycling conditions were one cycle of 94°C for 5 min, 55°C for 1 min, and 72°C for 90 s, then 31 cycles

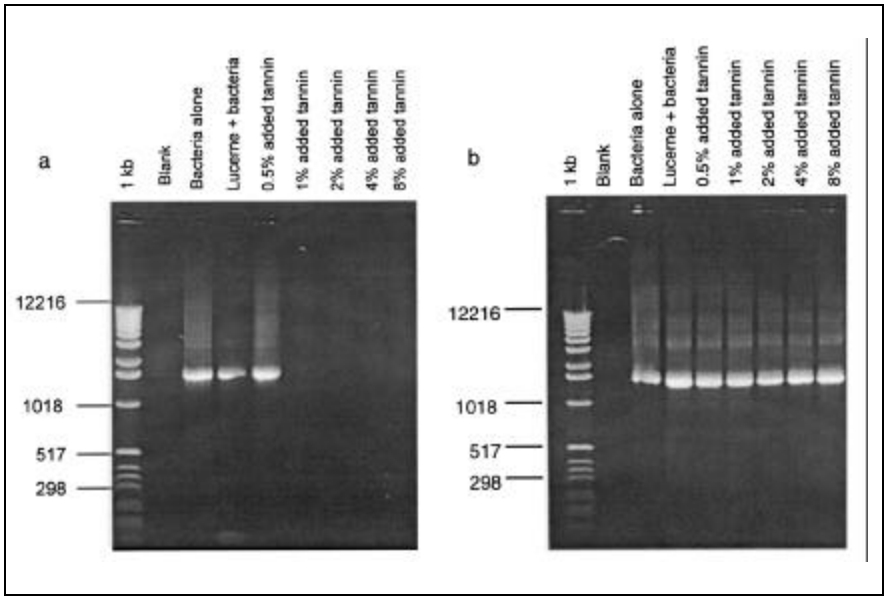

Figure 1. Effect of varying levels of quebracho tannin on the quality of extracted microbial DNA. Quebracho tannin was added to lucerne (tannin free) and mixed with rumen fluid rich in microorganisms. Microbial DNA was extracted from this mixture in the (a) absence or (b) presence of PEG, and the ability to obtain a 16S rDNA PCR product indicated whether the inhibitory effects of tannin on *Taq* DNA polymerase had been neutralized.

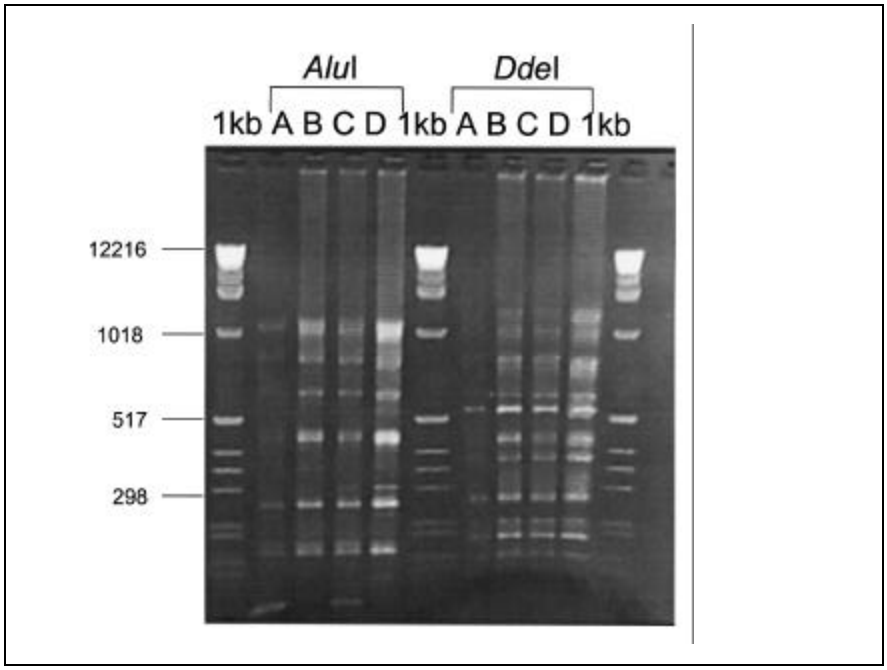

Figure 2. Microbial DNA was extracted (according to Table 1) from the rumen fluid of four sheep consuming a diet of 70% rhodes grass (*Chloris gayana*) and 30% calliandra (*Calliandra calothyrsus*). A 16S rDNA PCR product was obtained from extracted DNA restricted with (a) *Alu*I or (b) *Dde*I and run on an agarose gel to determine if the DNA was efficiently digested.

Table 1. Protocol for the Extraction of Microbial DNA from Rumen Fluid Containing Plant Tannins

1. Fill a 1.5-mL screw-cap conical vial with 0.5 g zirconium beads (75–200 µm), recap, and autoclave.
2. Dispense a bacterial-rich sample (e.g., rumen contents) into the screw-cap vial (100 mg wet weight). Centrifuge at 15000× *g* for 1 min and remove the supernatant.
3. Wash the pellet by adding 1 mL methanol and vortex mixing. Centrifuge at 15000× *g* and remove the supernatant.
4. Add 300 µL phenol:chloroform (1:1; phenol equilibrated with 100 mM Tris-HCl, pH 7.0), 200 µL ADEM buffer (50 mM sodium acetate, 10 mM Na_2-EDTA, 1% DMSO, 1% methanol, pH 7.0), and 100 µL 35% PEG solution [35% polyethylene glycol (M_r = 4000), 100 mM Tris-HCl, pH 7.0], and mechanically lyse the cells by bead-beating for 2 min.
5. Centrifuge the lysate at 15000× *g* for 5 min and retain the aqueous phase. Remove residual phenol with chloroform:isoamyl alchohol (24:1). Centrifuge for 10 min at 15000× *g* and transfer the supernatant to a fresh microcentrifuge tube.
6. Add 1/5 volume of 5 M NaCl and 1/10 volume of 10% CTAB to the supernatant and incubate at 65°C for 5–10 min. Centrifuge the sample at 15 000× *g* for 10 min to remove the polysaccharide precipitate.
7. Retain the supernatant and precipitate the DNA by adding 1/10 volume of 3 M sodium acetate plus an equal volume of 100% isopropanol.
8. Measure the concentration of DNA by gel electrophoresis.

of 94°C for 1 min, 55°C for 1 min, and 72°C for 90 s. The final PCR cycle was 94°C for 1 min, 55°C for 1 min, and 72°C for 8 min.

When the extraction protocol was used without PEG, PCR products could only be obtained at 0.5% and 1% added quebracho, and there appeared to be a sharp cut-off beyond this point (Figure 1a). This cut-off may be more gradual for quebracho levels between 1% and 2%, but we did not investigate this aspect. In contrast, a PCR product could be obtained at all levels of added tannin, even though there was a gradual decline in the intensity of the PCR product as the tannin level increased (Figure 1b).

A 100-mg sample of ground plant material from various shrubs containing tannin (lucerne with added calliandra, or quebracho tannin; Table 2) were mixed with 1 mL rumen fluid, and microbial DNA was extracted according to Table 1. A 16S rDNA PCR product could be amplified from all samples and was quantified by gel-densitometry (Quantity One®; Bio-Rad Laboratories, Hercules, CA, USA). The PCR and DNA yield and extraction efficiency of the rumen fluid control was greater ($P < 0.1$) than that of samples containing tannin (Table 2). Lucerne plus added tannin had PCR and DNA yields and an extraction efficiency that was less ($P < 0.1$) than rumen fluid alone, but greater ($P < 0.1$) than that of the rumen fluid plus tannin-containing plants (Table 2). The ranking between samples for the PCR and DNA yield assays were similar, but the PCR assay should not be considered definitive because it was not a truly quantitative assay as performed by Reilly and Attwood (19). These experiments indicate that the extraction protocol yielded high-quality DNA from a range of plants and that the polyphenols in these tannin-containing plants could be largely neutralized.

To ensure that the extraction protocol would perform efficiently, the method was tested under "field conditions" in which animals consumed a mixed diet and all the normal digestive processes of plant mastication, rumination, and fermentation would occur. DNA was extracted from rumen fluid obtained from four fistulated sheep, eating a diet consisting of 70% rhodes grass (*Chloris gayana*) and 30% *Calliandra calothyrsus* (Table 2). A 16S rDNA PCR product could be amplified, and 20 µL of the product could be digested with either *Dde*I or *Alu*I (Promega) at 39°C in the appropriate buffer for 2 h. The whole 20-µL digestion product was run on a 1% agarose gel, and the presence of restricted products, all smaller than uncut chromosomal DNA, indicated that the restriction enzymes were not being inhibited by tannin.

There are two hypotheses that may explain the inhibition of PCR by tannins: (*i*) the tannins bind and inactivate *Taq* DNA polymerase or (*ii*) the tannins alter the structure of DNA so that the primers cannot bind to the target. PCRs are inhibited by fat and protein (20), plant extracts (7), phosphate (24), and even humic substances (25). It is clear that tannins bind to protein (4) and enzymes (3,14), and this is the most likely means by which enzymes used in molecular biology are inhibited. To our knowledge, enzyme kinetics between *Taq* DNA polymerase and tannin have not been investigated, but kinetic data derived from inhibition assays of β-glucosidase by polyphenols indicate that tannins inhibit enzymes in a noncompetitive manner (18).

It is also possible that tannins modify the DNA target sufficiently to prevent the binding of the primers to the appropriate sites. It has previously been shown that small molecular weight phenolics, such as gallic and tannic acid, induce the degradation of DNA (21). The actual mechanism by which this occurs is not clear, but it appears as if tannic acid can result in the formation of reactive free radicals that alter the DNA, directly or indirectly, by strand breakage or even base modification (9). We used a methanol wash to remove phenolics before microbial cells were lysed and found that this procedure significantly increased the "shelf-life" of the extract (Table 1, step 3). This is presumably because high proportions of the small molecular weight phenolic compounds are removed, which would prevent free-radical formation and subsequent damage to the DNA.

We found that PCR and DNA yield were significantly ($P < 0.1$) lower when microbial DNA was extracted from rumen fluid plus lucerne than from rumen fluid alone (Table 2). Because lucerne does not contain tannins, it is possible that plant polysaccharide, or even lignin, bound the tannins and formed a complex that would decrease yield. Haslam (5) reviewed work that demonstrated

Table 2. PCR and DNA Yield and DNA Extraction Efficiency when Microbial DNA Was Extracted in the Presence of Plant Tannins

Item	[a]PCR	[b]DNA Yield (μg)	[c]Extraction Efficiency	[d]CT	Reference
Rumen fluid control (RF)	211[e]	78.9[e]	100.0	[h]NA	NA
Lucerne + RF	175[f]	67.3[f]	85.3	NA	NA
Lucerne + 5% extracted condensed calliandra tannin + RF	155[g]	55.6[g]	70.5	5	NA
Lucerne + 5% quebracho condensed tannin + RF	156[g]	56.8[g]	72.0	5	NA
Acacia angustissima CPI 66/92 + RF	133[g]	46.8[g]	59.3	5.9–6.6	1
A. angustissima CPI 15132 + RF	120[g]	47.8[g]	60.6	5.9–6.6	17
A. boliviana CPI 40175 + RF	117[g]	43.9[g]	55.6	2.4	6
A. villosa Q25222 + RF	123[g]	42.1[g]	53.4	6.0	12
A. villosa cv Indonesia + RF	134[g]	46.5[g]	58.9	6.0	12
Calliandra calothyrsus CPI 115690 + RF	115[g]	41.2[g]	52.2	5.7	15
Leucaena pallida CQ 3449 + RF	128[g]	43.6[g]	55.3	4.7	15
Leucaena diversifolia CPI 33820 + RF	100[g]	39.3[g]	49.8	7.7	15
Leucaena leucocephela cv Cunningham + RF	123[g]	44.1[g]	55.9	3.8	15

[a]PCR product was obtained with universal primers to the 16S rDNA (see text).
[b]DNA concentration was determined by running a sample on an agarose gel; band mass was determined with a densitometer.
[c]The efficiency of extraction was determined by comparison to the extraction yield of the rumen fluid control.
[d]CT, condensed tannin content as a percentage of dry matter, and their corresponding references are given. Quebracho tannin was obtained from Sigma (St. Louis, MO, USA), and the calliandra tannin was extracted as described by Terrill et al. (22).
[e,f, and g]Values in the same column with different superscripts are statistically different ($P < 0.1$).
[h]NA = not applicable.