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Rapid Identification of Rumen Protozoa by Restriction Analysis of Amplified 18S rRNA Gene

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Summary. A rapid method has been developed for molecular identification of rumen ciliates without the need for cultivation. Total DNA was isolated from single protozoal cells by the Chelex method and nearly complete protozoal 18S rRNA genes were amplified and subjected to restriction fragment length polymorphism analysis. On the basis of restriction patterns generated a molecular key was elaborated allowing identification of protozoa solely by a molecular technique without prior knowledge of morphology. No differences were observed between identical species originating from different animals or geographic locations, or between morphological variants of the same species. The ARDREA analysis described here provides a rapid and convenient way for identification and diversity studies of rumen protozoa.

Key words: 18S rRNA, ARDREA, ciliate, identification, protozoa, rumen.

Abbreviations: ARDREA - Amplified Ribosomal DNA Restriction Analysis, PCR - Polymerase Chain Reaction.

INTRODUCTION

Rumen protozoa represent a substantial part of the rumen microbial population contributing up to 50% of the total microbial biomass (Williams and Coleman 1992). However, due to highly specific growth requirement and their complex morphology our understanding of the role of protozoa in rumen fermentation is still limited. Ciliate protozoa in the rumen are classified on the basis of the micro- and macronucleus and the presence and morphology of exterior spines and lobes or internal skeletal plates as well as the shape and size of cells (Dogiel 1927, Ogimoto and Imai 1981, Williams and Coleman 1992). Based on such morphological characteristics a large number of genera and species have been described, but it is not clear to what extent these represent true species. Furthermore, morphological classification and identification of protozoa is made more complex as many of the morphological traits rapidly...
change or completely disappear under in vitro conditions (Williams and Coleman 1992). Molecular methods based on DNA analysis and fingerprinting provide a rational alternative to the classic morphology. In recent years the PCR amplification and sequencing of small subunit (SSU) rRNA genes from rumen bacteria and fungi have revolutionised studies on rumen microbial ecology. However, relatively few studies have applied this methodology to study the rumen protozoa (Hori and Osawa 1987, Lee and Kugrens 1992, Wright et al. 1997, Hirt et al. 1998), primarily due to the demanding growth requirement of rumen protozoa. The aim of this study was to develop a PCR fingerprinting method, which is independent of cultivation, for rapid identification of predominant rumen protozoa.

MATERIALS AND METHODS

Collection of protozoa. Twenty protozoal species were included in the study. The cultures came from monofaunated sheep obtained during the ERCULE project and species included (country of original place of isolation is shown in parentheses) Dasytricha ruminantium (France), Diplodinium dentatum (Poland), Diplodintron affine (Poland), Enoploplastron triloricatum (Poland), Entodinium bursa (Slovakia), Ent. caudatum (Scotland and Slovakia), Ent. furca monolobum (Slovakia), Ent. nanellum (Slovakia), Ent. simplex (Poland), Epidinium ecuadatum (Poland), Euodiplodinium maggii (France and Poland), Isotricha intestinalis (Poland), I. prostoma (France and Slovakia), Metadinium medium (Poland), Ophryoscolex caudatus (Poland), Oph. purkynjei (Poland), Ostracodinium gracile (Poland), Oste. dentatum (Poland), Polyplostron multivesiculatum (France and Poland). For study of protozoal variability Oph. caudatus (forma ricorumatus) cells were directly picked out from fresh rumen fluid of goat and sheep from Slovakia. The morphological variants of Dipl. dentatum were picked from an in vitro culture kept in Kielanowski Institute of Animal Physiology and Nutrition of Jablonna. Entodinium caudatum type and forma dubaridii cells were from an in vitro culture kept in Institute of Animal Physiology, Kosice, Slovakia.

DNA isolation and analysis. A single protozoal cell was picked either from rumen fluid or from in vitro culture under the microscope, washed twice in drop of sterile water and put into 50 µl of 5% Chelex-100 (BioRad, California, USA) in water. Pre-incubated proteinase K (Merck, Germany) was then added to the reaction mixture to a final concentration of 20 µg/ml. After proteinase treatment (55°C for 30 min) DNA was released from cell by heating the sample at 98°C for 5 min. After rapid cooling to 0°C and centrifugation (3000g for 5 min) DNA containing supernatant was directly used for PCR amplification. All isolation and manipulation steps were done under aerobic conditions.

Polymerase chain reaction. Five µl of isolated DNA was amplified using a Techne Thermal Cycler Progene in a 50 µl reaction mix containing 0.04 mM of each deoxynucleoside triphosphate, 20 pmol of each primer, PCR reaction buffer (Perkin Elmer), and 0.5 U of AmpliTaq DNA polymerase (Perkin Elmer). An initial denaturation step at 95°C for 5 min was followed by 35 cycles of (94°C for 1 min, 52°C for 1 min and 72°C for 1 min), and a final incubation at 72°C for 10 min. Primers based on conserved regions in eukaryotic 18S rDNA genes were used in the PCR amplification: EukFor (5’-AAATAGTTGATCTTGCGACAGT-3’) and EukRev (5’-TGATCTCTTGGAGTTACCTAC-3’). Quality and quantity of amplified DNA was determined by electrophoresis in 1 % agarose gel (Maniatis et al. 1982).

Amplified DNA (about 0.5 µg per reaction) was digested by 18 restriction endonucleases - AccI, AseI, AvaII, BanII, BsrUI, CfoI, EcoRV, HaeIII, HindIII, MluI, MspI, NruI, SacII, SpeI, Stul, StyI and XmaI (New England Biolabs and Gibco BRL), according to manufacturer’s instructions for 1 h. Digestion products were separated by horizontal 2% agarose gel electrophoresis in Tris-acetate buffer (Maniatis et al. 1982).

RESULTS

A rapid method has been developed for molecular identification of rumen protozoa without the need for cultivation. Total DNA was isolated from single protozoal cells by the Chelex method and used as a target for PCR amplification using primers directed to the 18S rDNA gene. The nearly complete SSU rDNA gene was obtained by PCR amplification from all tested samples. Amplified DNA was then subjected to cleavage by several (18) restriction endonucleases recognizing tetra- or hexa-nucleotide sequences and fragments generated were resolved by agarose gel electrophoresis. Specific DNA fingerprints were obtained after agarose gel electrophoresis (Fig. 1). While for example all tested species produced identical banding patterns after cleavage by BanII and CfoI restriction endonucleases, substantial variability was observed after the cleavage by AccI (four different profiles) or AseI endonuclease (five different profiles). The highest discriminatory power was observed for MspI and BsrUI endonuclease (six profiles). The restriction fragment length polymorphism (RFLP) analysis of amplified 18S rDNA genes was found to clearly discriminate between all species studied, with DNA from each species giving unique sets of patterns. The fingerprints were recorded and species were grouped into similarity groups. The data obtained from DNA cleavage were then correlated with data obtained by computer-aided analysis of available 18S rDNA sequences from GenBank and a molecular key was designed. Identification starts by AvaII cleavage and by using six selected endonucleases any from 20 protozoal species be can unambiguously identified (Table 1).
No differences were observed between identical species originating from different animals or geographic locations, or between morphological variants of the same species. Analysis of animal-to-animal variability did not reveal any variability in *Ophryoscolex caudatus f. tricoronatus* (Figs 2A, B). No differences were found in morphologically different *Diplodinium dentatum* strains (data not shown) and similarly no differences were found in several geographic and morphological invariability of ARDREA profiles of *Ophryoscolex caudatus f. tricoronatus* strains isolated from Slovak sheep - lane 1, Slovak goat - lane 2 and Polish sheep - lane 3 obtained by BsrUI (part A) andMspI (part B) restriction endonuclease. Part C - ARDREA profiles of *Entodinium caudatum* type strains (lanes 1-3) were compared to those of *f. dubardii* (lanes 4-7) obtained by BsrUI restriction endonuclease. Lane M - 100 bp DNA ladder (Gibco BRL, California USA), standard of molecular weight. The arrow indicates marker band of 600 bp.

Fig. 1. Schematic representation of ARDREA profiles of selected protozoal species obtained by AvaII (lane 1), AseI (lane 2) and MspI (lane 3) restriction endonucleases.

Figs 2A-C. Geographic and morphological invariability of ARDREA profiles of *Ophryoscolex caudatus f. tricoronatus* strains isolated from Slovak sheep - lane 1, Slovak goat - lane 2 and Polish sheep - lane 3 obtained by BsrUI (part A) and MspI (part B) restriction endonuclease. Part C - ARDREA profiles of *Entodinium caudatum* type strains (lanes 1-3) were compared to those of *f. dubardii* (lanes 4-7) obtained by BsrUI restriction endonuclease. Lane M - 100 bp DNA ladder (Gibco BRL, California USA), standard of molecular weight. The arrow indicates marker band of 600 bp.
Table 1. Key for RFLP analysis of rumen protozoa.

<table>
<thead>
<tr>
<th>Digestion with</th>
<th>Fragments</th>
<th>Species/Strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ava II</td>
<td>(1080, 550)</td>
<td>Entodinium sp.</td>
</tr>
<tr>
<td>Nru I</td>
<td>(1100, 450)</td>
<td>Ent. bursa</td>
</tr>
<tr>
<td>Msp I</td>
<td>(1530, 100)</td>
<td>Ent. nannellum</td>
</tr>
<tr>
<td>Acc I</td>
<td>(920, 610, 100)</td>
<td>Ent. caudatum</td>
</tr>
<tr>
<td>Msp I</td>
<td>(920, 610, 100)</td>
<td>Ent. caudatum</td>
</tr>
<tr>
<td>Acc I</td>
<td>(750, 600, 300)</td>
<td>Ophryoscolex sp.</td>
</tr>
<tr>
<td>Nru I</td>
<td>(1100, 450)</td>
<td>Epidinium sp.</td>
</tr>
<tr>
<td>Msp I</td>
<td>(1530, 100)</td>
<td>Epid. caudatum</td>
</tr>
<tr>
<td>Acc I</td>
<td>(750, 600, 300)</td>
<td>Enoploplastron triloricatum</td>
</tr>
<tr>
<td>Nru I</td>
<td>(1100, 450)</td>
<td>Epid. caudatum</td>
</tr>
<tr>
<td>Msp I</td>
<td>(1330, 200, 100)</td>
<td>Ophr. purkynjei</td>
</tr>
<tr>
<td>Acc I</td>
<td>(1350, 300)</td>
<td>Ophr. caudatum</td>
</tr>
<tr>
<td>Msp I</td>
<td>(1330, 200, 100)</td>
<td>Epid. caudatum</td>
</tr>
<tr>
<td>Acc I</td>
<td>(1350, 300)</td>
<td>Dasytricha ruminantium</td>
</tr>
<tr>
<td>Nru I</td>
<td>(1100, 450)</td>
<td>Isotricha prostoma</td>
</tr>
<tr>
<td>Msp I</td>
<td>(1350, 300)</td>
<td>Ostracodinium dentatum</td>
</tr>
<tr>
<td>Acc I</td>
<td>(1350, 300)</td>
<td>Ostracodinium gracile</td>
</tr>
</tbody>
</table>

Note: Numbers in parentheses refer to fragment sizes in base pairs.
morphotypes of *Ent. caudatum* (Fig. 2C), indicating that morphological variability observed is not due to genetic heterogeneity.

**DISCUSSION**

The rumen ciliates are the most abundant protozoa in the rumen and are involved in host metabolism and digestion of plant material. By classical morphological criteria more than 250 species of ciliates have been described which live in the rumen of various feral and domesticated ruminants (Williams and Coleman 1992). Identification of protozoa by these criteria is extremely tedious and requires extensive special knowledge and skills. Moreover, the validity of classical identification is questioned, since many of the "species" described exhibit a substantial morphological plasticity (Dehority 1994). Introduction of modern molecular methods based on DNA analysis and fingerprints, especially the methods targeted at ribosomal RNA operon provide exact insight into similarity studies of micro-organisms. Recent research using molecular characterisation has suggested that the protozoal diversity within the rumen is even greater than that first anticipated, but despite recent progress with molecular ecological studies (Karnati et al. 2003), the level of diversity present between individuals remains unclear.

While there are numerous examples of application of molecular methods for identification of bacteria (e.g. Blanc et al. 1997), only a few papers deal with identification of protozoa (Yang et al. 2002). The primary objective of the present work was to develop a rapid method for identification of predominant rumen protozoa. Restriction analysis of amplified ribosomal DNA method described here was found to be able to clearly discriminate between all strains studied. Use of the Chelex method enabled isolation of DNA from single protozoal cell thus reducing possible contamination by foreign DNA from feeds or fungi. On the basis of DNA fingerprints obtained a molecular key was designed. While all 18 restriction endonucleases provided some discrimination between strains, the key was optimized in order to minimize the number of cleavage steps and endonucleases used. While small *Entodinium* could be identified in four steps, up to six restriction endonucleases cleavage steps had to be used to unequivocally identify *Ostr. dentatum* and *Diplod. dentatum* species. RFLP patterns were found to be stable and reproducible. In addition, to providing a simple method for discrimination of morphologically similar species, the RFLP technique also demonstrated that new morphologically different variants (e.g. Dehority 1994) are not necessarily new species. No differences were observed between identical protozoal species originating from different animals or geographic locations, or between morphological variants of the same species, indicating limited intra-species variability of studied protozoa. These data are in correlation with a previous report on very limited intra-species sequence variation among eight isolates of the rumen ciliate *I. prostoma* (Wright 1999).

In conclusion, the methodology and molecular key described here provides a rapid and convenient way for identification of rumen protozoa. Furthermore, it enables the examination of the diversity of rumen protozoa without requiring specialist knowledge regarding the morphological characteristics of the ciliates being studied. The technique may be performed in even a basic molecular laboratory and by a researcher unskilled in the identification of rumen protozoa by traditional means.

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