ISNN 1517-6770

Improved silver carbonate impregnation method for rumen ciliate protozoa

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Abstract. This study proposes improvements to the pyridinated silver carbonate impregnation technique in rumen ciliate protozoa in order to provide a standardized impregnation protocol for usable for the largest possible number of species in domestic ruminants. The proposed improvements are based on results obtained from impregnation of oral infraciliature and nuclear apparatus of 36 rumen ciliate species, which are symbionts of domestic ruminants. Compared to established protocols for morphology of rumen ciliates, impregnation of a wider range of genera and species was observed with the proposed protocol. The impregnation time varied according to size, ciliate taxon, or both, varying from shorter (5 minutes) for small entodiniomorphid ciliates (<80 µm) and the genus *Dasytricha*, to longer (30 minutes) for large entodiniomorphid ciliates (>80 µm) and the genus *Isotricha*. The proposed protocol is simple and easily reproducible. It is also advantageous for taxonomic, animal science, and ecological studies that aim to inventory the ruminal biota as well as understand the population structure of rumen ciliates and their relationship with the host.

Keywords: ciliatological techniques, Ciliophora, oral infraciliature, silver impregnation.

Introduction

The use of silver impregnation techniques is essential for the taxonomic study of almost all groups of ciliated protozoa (Klein, 1958; Wilbert, 1975; Montagnes & Lynn, 1987; Roberts & Causton, 1988; Foissner, 1991). Several studies have recommended its use for visualizing structures important for the correct identification and description of rumen ciliate protozoa (Tuffrau, 1967; D'Agosto & Santa-Rosa, 1994; Cameron *et al.,* 2000).

The pyridinated silver carbonate impregnation technique was described by Fernandez-Galiano (1966) and is used to visualize the infraciliature and nuclear apparatus in different ciliate species (Augustin *et al*., 1984; Foissner, 1991, 1992; Ito & Imai, 1998, 2000, 2003, 2006; Ito *et al.*, 2001, 2006, 2008, 2011; Ma *et al*., 2003). For rumen ciliates, this technique provides excellent results by revealing oral and somatic infraciliatures (Ito *et al.,* 2001; Ito & Imai, 1998, 2003, 2006; Mishima *et al.*, 2009). According to Foissner (1991), large amounts of ciliates are required to obtain good results with the silver carbonate

technique. Therefore, studies with rumen ciliates are advantageous since a ruminants host a high abundance and diversity of ciliate species.

The organization of somatic ciliature and especially of oral ciliature varies considerably among ciliate species. It is therefore important to document the oral ciliature of species in detail, considering their specific characteristics. The present study aims to evaluate the existing pyridinated silver carbonate impregnation protocols for rumen ciliates, proposing a simple, more efficient protocol that allows for impregnation of the largest possible number of species.

Material and Methods

Collection and processing of samples

Samples of rumen contents were collected from a Holandês-Gir cow and a Morada Nova sheep, both of which had a fistula in the rumen. Samples were obtained from the center of the rumen via the rumen fistula, and stored at 39°C. Subsequently, rumen contents were filtered through a double gauze layer, disposed in a 15 ml Falcon tube, and centrifuged at 1300 rpm for 5 minutes. The supernatant was discarded and the pellet of each sample was used for the silver impregnation technique.

Pyridinated silver carbonate impregnation technique

Proposed improvements to the pyridinated silver carbonate impregnation technique were based on analysis of various protocols used for different ciliate groups (Fernandez-Galiano, 1976), specifically for rumen ciliates (Ito & Imai, 1998; 2006). Information from analysis of these protocols was used to prepare a simplified protocol that would be efficient for impregnating the largest possible number of species. The improvements were primarily implemented in the fixation stage of ruminal content, with addition of Rio Hortega solution, and in the incubation time of the sample in the water bath. The Ito & Imai (2006) protocol was followed for other preparation steps and addition of other reagents. The sample centrifugation step was tested before and after fixation of ruminal content. In order to reduce the protocol implementation time, the post-incubation step was suppressed, as proposed by Fernandez-Galiano (1976). The protocol optimized in this study is detailed below:

Improved silver carbonate impregnation protocol

1) Centrifuge fresh or previously formalin-fixed (18.5% v/v) samples of ruminal contents at 1300 rpm for 5 minutes.
2) Fix the sample precipitates with formalin (18.5% v/v) (Dehority, 1984).
3) In a test tube, add 3 drops of the fixed sample (after centrifugation) and 4 ml of distilled water.
4) Add the following reagents sequentially to the test tube at room temperature: 4 drops of pyridine PA, 6 drops of 4% bacteriological proteose peptone, and 40 drops of Rio Hortega solution (Fernandez-Galiano, 1976).
Remark: The solution will become milky white at this stage.
5) Incubate the test tube in a water bath at 40 °C for about 30 minutes.
Remark: After a few minutes of incubation, the solution will become transparent brown and then opaque brown, indicating silver impregnation.
6) After 30 minutes of incubation, observe ciliates between slide and cover slip under light microscopy.

Remark: In small entodiniomorphids and *Dasytricha*, oral and somatic infraciliature was impregnated after 5 minutes of incubation, whereas in large entodiniomorphids and *Isotricha*, impregnation of oral and somatic infraciliature was observed after 30 minutes of incubation. In small entodiniomorphids *Dasytricha*, oral and somatic infraciliature remain well impregnated after 30 minutes of incubation. Permanent slides were prepared according to the methodology proposed by Ito & Imai (2006).

Results

The main features visible in rumen ciliates impregnated by ammoniacal silver carbonate were oral and somatic ciliature and the nuclear apparatus. These features were noted in 36 rumen ciliate species after applying the new protocol established in this study.

In Figure 1, we show successful silver impregnation of representatives of the families Ophryoscolecidae and Isotrichidae, showing the oral and somatic infraciliature and the nuclear apparatus in detail. The first protocol modification occurred in the use of 18.5% formalin for sample fixation (Dehority, 1984). This preserved ciliate morphology without changing the impregnation process. Second, we assessed whether centrifugation of ciliates was optimal before or after the fixation step. No significant morphological changes or differences in the ciliate impregnation process were observed when centrifugation was performed either before or after sample fixation.
Among the reagents used in the new protocol, a proportionately increased amount of 40 drops of Rio Hortega solution was required for impregnation of oral and somatic infraciliatures.

In the water bath incubation step, incubation at 40°C for 30 minutes proved most successful for impregnation of any ciliates, whereas impregnation was not observed at temperatures below 40°C. Oral infraciliature and the nuclear apparatus showed excellent impregnation at temperatures ranging from 40°C to 7°C, with unsatisfactory results at temperatures above 70°C.

It was observed that the incubation time for successful impregnation depended on the size and ciliate genus. For small entodiniomorphids (Figure 1e-i) and the genus *Dasytricha* Schuberg, 1888 (Figure 1a), oral and somatic infraciliature was impregnated after 5 minutes, while for large entodiniomorphids (Figure 1j-l) and the genus *Isotricha* Stein, 1859 (Figure 1b-d), impregnation of oral and somatic infraciliature occurred after 30 minutes. However, after 30 minutes of incubation, all the ciliate groups used showed excellent impregnation of oral and somatic infraciliature and the nuclear apparatus could be photographed under an optical microscope.

Discussion

Aspects of oral and somatic infraciliature have been widely used in morphological characterization of rumen ciliates (Ito & Imai, 1998, 2003, 2005; Ito *et al*., 2001; Mishima *et al.*, 2009). The impregnation of oral and somatic infraciliature in several groups of ciliates has been made possible with the ammoniacal silver carbonate technique, which has been modified over the years by several authors (Fernandez-Galiano, 1976; Augustin *et al*.; 1984; Foissner, 1991, 1992; Ito & Imai, 1998, 2003, 2006; Ito *et al*., 2000, 2001, 2006, 2008, 2011; Ma *et al*., 2003). However, the principal methodologies used for the silver carbonate impregnation of rumen ciliates are based on proposals from Fernandez-

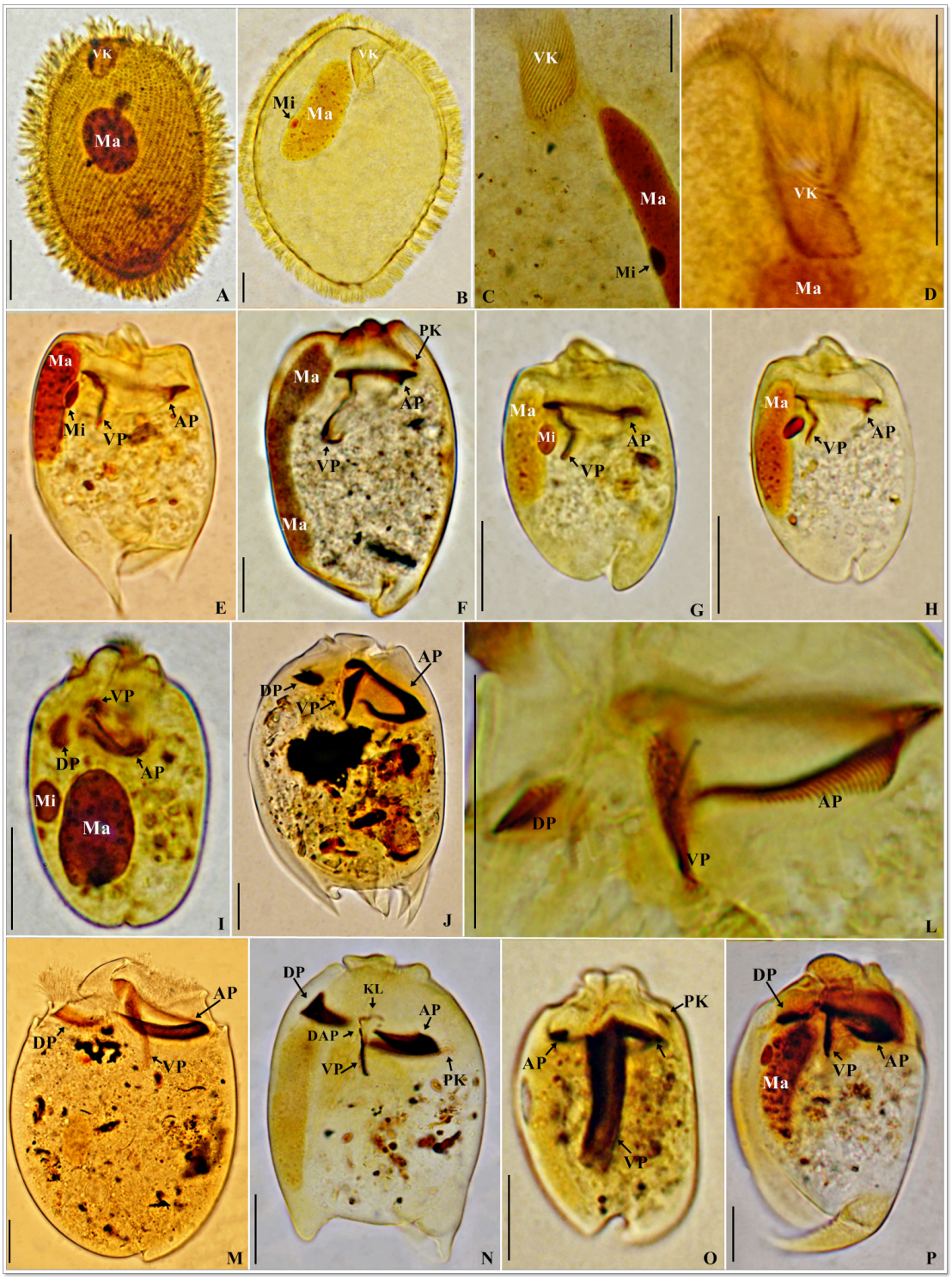

Figure 1. Rumen ciliates after pyridinated silver carbonate impregnation. **A.** *Isotricha prostoma;* **B.** *Dasytricha ruminantium*; **C-D.** *Isotricha prostoma*. **E.** *Entodinium caudatum lobosospinosum*; **F.** *Entodinium longinucleatum longinucleatum*; **G.** *Entodinium*; **H.** *Entodinium*; **I.** *Diplodinium polygonale*; **J.** *Diplodinium anisacathum*; **K.** *Diplodinium anisacanthum* **L.** *Polyplastron multivesiculatum*. **M.** *Ostracodinium mammosum*; **N.** *Eodinium posterovesiculatum bilobosum*. **O.** *Eremoplastron rostratum*. **AP.** Adoral polybrachykinety; **DAP.** Dorso-adoral polybrachykinety; **DP.** Dorsal polybrachykinety; **KL.** Kinety loop; **Ma:** Macronucleus; **Mi:** Micronucleus; **PK.** Paralabial kineties; **VK.** Vestibular kineties; **VP.** Vestibular polybrachykinety. Scale bars: 20 µm.

Galiano (1976) and Ito & Imai (1998, 2006).

The revised protocol presented in this study allows impregnation of the oral infraciliature and the nuclear apparatus in a broad spectrum of rumen ciliate species from different hosts. Among the changes made, fixation of samples from rumen contents with 18.5% formalin (Dehority, 1984) allows for the preservation of ciliates without interfering with the final result of the technique, and provides the same efficacy as in protocols, which use formalin PA (Fernandez-Galiano, 1976), 10% formalin and methyl-green formalin saline (MFS) solution (Ito & Imai, 1998; 2006). In addition, the use of 18.5% formalin is advantageous, since this fixative is commonly used in ecological and animal science studies for evaluating the role of environmental effects on the composition and dynamics of ciliate protozoa populations (Fondevila & Dehority, 2001; Wang *et al*., 2008; Martinele *et al*., 2010; Talebzadeh *et al*., 2012; Gurelli *et al*., 2012; Fiorentini *et al*., 2013). Thus, samples fixed with a single fixative, 18.5% formalin in this case, could be used for different purposes, including taxonomic studies.

In the present study, the centrifugation step was tested before and after fixation of the samples; however, there were no significant morphological differences in ciliates or the impregnation process (Ito & Imai, 1998; Ito *et al.*, 2006). In agreement with established protocols, we consider centrifugation after sample fixation as the best alternative, as shown in the sample cleaning at the end of the process.

The protocol present in this study showed a more flexible incubation temperature range than other protocols, with satisfactory impregnation between 40°C and 70°C. However, the incubation temperatures proposed by other rumen ciliate protocols vary between 60°C and 70°C (Fernandez-Galiano, 1976; Ito & Imai, 1998; Ito *et al.*, 2006). This difference may be related to the wide range of species analyzed in this study and their bodily differences.

When adapting the silver carbonate impregnation technique for marine ciliates, Ma *et al*. (2003) observed that the required amount of Rio Hortega solution depended on the group and size of the organism. In the present study, the Rio Hortega solution was modified with respect to the number of drops used. At least 40 drops of the solution were required for effective impregnation of ciliates. The increased amount of Rio Hortega solution used in the proposed protocol provides an advantage over other protocols because satisfactory impregnation of the oral and somatic infraciliature and nuclear apparatus of all ciliate species found in the cattleand sheep rumen samples analyzed was obtained with this volume. The incubation time for this group varied according to the size and habitat of the organism under study (Ma *et al*., 2003). The longest incubation time for the larger size ciliates may be related to the presence of a thicker film (Noirot-Timothée, 1960), which made the penetration of reagents for impregnation of the infraciliature and nuclear apparatus difficult. Despite the variation in incubation time in relation with ciliate size, the infraciliature and nuclear apparatus of all species were impregnated after 30 minutes, which suggests that it can be considered a standard time for satisfactory impregnation of all groups of rumen ciliates.

The proposed protocol is simple, reproducible, and allows for wider coverage than that established by Fernandez-Galiano (1976) and Ito & Imai (1998, 2006). The practicality of this protocol allows its use in different disciplines, unlike

other laborious techniques for silver impregnation. This new protocol is therefore advantageous for taxonomists interested in ruminal biota, as well as for animal scientists and ecologists interested in understanding the structure of ciliate protozoa assembly.

Acknowledgements

This work was partially supported by INMETRO (Estudo do Processo de Degradação de Biomassa por Microrganismos do Rúmen). We thank Luisa Oliveira for helping in laboratory work, CNPq (Bolsa de Produtividade PQ) for grants to Marta D'Agosto and Roberto Dias and CAPES for grants to Isabel Martinele, Mariana Rossi and Franciane Cedrola.

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