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Note

Alternative mounting media for preservation of some protozoa

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ABSTRACT

Protozoa resistant stages are disintegrated when mounted in toluene-based media. To overcome such problem, three toluene-free mountants were tested on preserve *Acanthamoeba* spp and gregarines. Two commercial glues based on cyanoacrylate or trimethoxysilane were suitable for preserving both cysts and trophozoites. Hoyer's medium showed good results for mounting gregarine oocysts.

Protozoan dormant stages are important structures in morphological studies, since they are relatively uniform in size and shape (Janovy et al., 2007). *Acanthamoeba* spp. (Sarcodina, Acanthopodida) present a characteristic double-walled cyst with pores which permits identification of amoeba to genus level under microscopic observation. Moreover, cyst morphology was considered essential to define the four groups present in the former classification system of *Acanthamoeba* (Rondanelli, 1987). In recent years, molecular studies have shown that classification and diagnosis of amoeba species within this genus should not be exclusively based on morphology and analysis of the 18S rRNA gene is recommended for identification (Schroeder et al., 2001). In gregarines (Apicomplexa, Eugregarinorida) oocysts are likewise considered a cardinal phase in taxonomy, due to their distinct and consistent morphology (Clopton et al., 1991). Both in amoebae and gregarines resting stages are seldom included as permanent type specimens (hapantotypes) in reference collections (Clopton et al., 1992; Ledee et al., 1996; Johny and Withman, 2005; Clopton and Hays, 2006; Qvarnstrom et al., 2013). Nevertheless, voucher specimens deposited in natural history collections are essential in order to identify species used in biological studies and a requirement for erection of new taxa according to ICZN regulations. By these reasons, a study on preservation of resistant stages of protozoa with alternative mounting media has been developed. Trophozoites were included as part of the investigation. Three protozoa species were employed as models in this research: two facultative pathogens (*Acanthamoeba astronyxis* and *Acanthamoeba griffini*) and a parasite (*Gregarina* sp.). The Hoyer's medium and two commercial glues, transparent with refractive indices similar to glass were tested.

Two *Acanthamoeba* species available in our laboratory were selected for preservation experiments: *A. astronyxis* CCAP 1501/9, a type strain species chosen because it presents trophozoites with numerous acanthopodia and *A. griffini* MYP2004, which shows a characteristic star-like endocyst (Heredero-Bermejo et al., 2014). Amoeba culture conditions and harvesting techniques were performed according to Heredero-Bermejo et al. (2014) and Lorenzo-Morales et al. (2008).

Gregarines were obtained from *Scaurus punctatus* Fabricius 1789 (Coleoptera, Tenebrionidae) beetles, which were captured by pitfall traps in the immediate vicinity of the Facultad de Farmacia at the University of Alcalá (Madrid-Spain). Protozoa trophozoites were fixed to microscopic slides in squash preparations of intestine, whereas gametocysts were recovered in feces for use either in preservation or for obtaining oocysts. Gametocysts were deposited on a microscopic glass slide in a drop of sterile saline solution (0.9% NaCl) and kept in a moist chamber at 24 °C for 2–5 days. After gametocyst dehiscence oocysts emitted in chains remained adhered to the glass slide and then were used for permanent microscopic slides preparation.

Canada balsam (Sigma, St Louis, MO, USA) was used as standard mounting media. Two commercial glues (Loctite® — Henkel Ibérica, Barcelona, Spain), Super Glue® (ethyl-cyanoacrylate) and Hybrid Glue® [(3-(2-aminoethyl) aminopropil) trimethoxysilane], were tested as alternative mounting media. Hoyer's medium was used for mounting resistant stages only. It was prepared as described by Moreno and Manjón (2010).

For permanent mounting of amoebae, 150 μl of *Acanthamoeba* spp. trophozoites (exponential phase culture, 3–4 days old) were deposited

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on 8 mm circular cover glass and incubated for 2 h at 24 °C in a moist chamber to let them adhere to the glass surface. The protozoa were then fixed in 2% glutaraldehyde for 2 h, washed in saline for 2 min, stained for 5 min with 0.1% toluidine blue. The excess stain was washed off with distilled water. Protozoa trophozoites were then dehydrated in an ethanol series (70%, 85%, 96% and three times in 100% ethanol, 2 min for each step). Fixed protozoa in coverslips were then mounted following standard procedure (Canada balsam) or commercial glues. In the alternative method, amoebae were dried in air at 37 °C for 10 min before mounting with adhesive. On the other hand, amoeba cysts were harvested by centrifugation of 10 ml of encystment medium (EM) containing resistant stages for 10 min at 1000 ×*g*. These were fixed with 2% glutaraldehyde, washed in saline and resuspended in 0.1% L-polylysine at a density of about 100 protozoa per μl. Twenty μl of cyst suspension were deposited on glass slides and allowed to dry at ambient conditions. Cysts were subsequently dehydrated in ethanol, dried and mounted as explained for trophozoites. Alternatively, the pellet obtained after centrifugation of EM was resuspended in distilled water. Twenty μl of cyst suspension were deposited on a glass slide and allowed to dry directly (no addition of polylysine and dehydration) before mounting.

For permanent mounting of gregarines, trophozoites from insect's intestine squash preparations were fixed to microscopic glass slides by treatment with AFA (alcohol:formalin:acetic acid — 1:1:1), whereas gametocysts were treated with the same fixative in Eppendorf tubes. Both trophozoites and gametocysts were stained with either acetocarmine (Semichon, 1924) or eosine-Y (1.22 g/l, Diff-Quick® Stain solution I). After staining, protozoa stages were either dehydrated with ethanol and toluene and mounted with Canada balsam (standard method) or (alternative method) dehydrated in ethanol series of 70%, 85%, 96% and absolute ethanol, a minimum of 5 min for each step and finally allowed to dry for 10 min at 37 °C before mounting with glue. Oocyst mounting was performed directly without fixation. Alternatively, oocysts were fixed on microscopic slides with 2% glutaraldehyde for 2 h and then rinsed in saline. One drop of 0.1% L-polylysine was added to the preparation and the liquid was allowed to dry. Once oocysts were coated with polylysine, they were dehydrated and dried following the method explained above for *Acanthamoeba* cysts.

Cysts and oocysts intended for preservation in Hoyer's medium were not dehydrated but directly mounted. After one month, preparations of resistant stages were sealed around the cover glass ("ringed") with acrylic medium 012 (Talens, Apeldoorn, Holland). Ringing ensures stability of specimens for long term storage, as recommended by Moreno and Manjón (2010). Processing of dormant stages for Scanning Electron Microscopy (SEM) was performed following the method by Heredero-Bermejo et al. (2014) for *Acanthamoeba* sp. and according to Janovy et al. (2007) for gregarines. The ultrastructural method was only used for resistant stages. All microscopic slide preparation procedures for the same protozoan samples were done in triplicates.

Fig. 1 shows a comparison of different protozoa stages in wet mounts versus the resulting features of the same, preserved for one year in different mounting media and the dormant stages as observed by SEM. Commercial glues cyanoacrylate and trimethoxysilane showed satisfactory results for amoeba trophozoites (Fig. 1, 1C and D), with well preserved acanthopodia (Fig. 1, 1B). Untreated *A. griffini* cysts disintegrated with toluene (Canada balsam) and cyanoacrylate. However, cysts coated with polylysine were not affected by addition of cyanoacrylate, although their preservation was suboptimal (Fig. 1, 2B) as compared to addition of trimethoxysilane only (Fig. 1, 2C; refer to wet mount 2A and SEM micrograph 2E). Cysts mounted in Hoyer's medium were transparent and the star-like endocyst was difficult to observe (Fig. 1, 2D). Carmine-stained gregarine trophozoites mounted in cyanoacrylate (Fig. 1, 3C) were relatively well preserved and looked similar to live or Canada Balsam-mounted protozoa (Fig. 1, 3A and 3B). Preservation in trimethoxysilane of carmine-stained gregarines was not advisable, since blackening of specimens was observed eventually (data not shown). Such artifact was not seen in eosine-stained protozoa (Fig. 1, 3D). Gregarine gametocysts retained their outer mucilaginous layer when mounted with both glues (Fig. 1, 4C and 4D), in contrast with the standard procedure using toluene, where it was deformed or lost (Fig. 1, 4B). In the same manner as for *Acanthamoeba* cysts, untreated gregarine oocysts were totally disintegrated with toluene (Canada balsam) or cyanoacrylate. However, resistant stages treated with polylysine were relatively well preserved in cyanoacrylate (Fig. 1, 5B). Addition of polylysine was not necessary for successful mounting in trimethoxysilane (Fig. 1, 5C). Oocysts in wet mount (Fig. 1, 5A) were dolioform in shape and maintained a dorsoventral position, but when preserved in glue some changed to a lateral position. In this case their shape was rectangular with blunt ends, the same as observed by SEM (Fig. 1, 5E). Finally, gregarine resistant stages mounted in aqueous Hoyer's medium showed dolioform shape (Fig. 1, 5D) like in wet mount preparations (Fig. 1, 5A).

As far as we know, this investigation is the first to describe new methods for permanent mounting of resistant stages of *Acanthamoeba* and gregarines. Moreover, the techniques could be applied as well for trophozoites. The refraction index (RI) of any mounting media should be close as possible to that of glass (RI = 1.5), according to Ravikumar et al. (2014). Hoyer's medium (RI = 1.48), cyanoacrylate (RI = 1.45) and trimethoxysilane (RI = 1.45) comply such criterion. Commercial adhesives are comparable to some standard mountants. Loctite® UV 358 has been used for the preparation of microscopic slides in museums (Brown, 1997). Likewise, cyanoacrylate glue has been reported as an lternative mounting medium for resin-embedded semithin sections (Liu et al., 2010).

Some authors who described new *Acanthamoeba* species failed to deposit hapantotypes in museum's reference collections (Ledee et al., 1996; Qvarnstrom et al., 2013). Therefore, the erection of these species is incomplete from the zoological standpoint. Such a problem may be solved with the new microscopic mounting media described in the present paper. The same is true for gregarine resistant stages. When glue was used as mounting medium, the morphology of *Gregarina* sp. oocysts was similar to that seen for *Gregarina polymorpha* or *Gregarina cuneata* resistant stages in glycerin mount (Clopton et al., 1991). In contrast, oocysts preserved in Hoyer's medium showed similar aspect than those in wet mount, confirming its suitability for mounting parasites, as previously reported by Cielecka et al. (2009). The new procedures described here are likewise important for gregarine taxonomy, as the usual hapantotypes deposited in reference collections consist only in trophozoites, gamonts or associations (Clopton et al., 1992; Johny and Withman, 2005; Clopton and Hays, 2006). However, various authors have pointed out that oocysts are morphologically informative because of their consistent structure (Clopton et al., 1991; Janovy et al., 2007).

Unringed microscopic slides made with Hoyer's medium are considered as semipermanent (Brown, 1997), whereas ringed (sealed) preparations are permanent, with a storage record of more than 40 years (Moreno and Manjón, 2010). As observed in our study, microscopic slides prepared with alternative mounting media for a year up to now remained in good condition. Of course this is not comparable with the archival quality of Canada balsam as standard mountant being used perfectly for more than a century (Ravikumar et al., 2014). Brown (1997) pointed out that a conservative approach should be envisaged by museum curators, employing always traditional permanent mounting media for preservation. Notwithstanding, such idea should not apply to research institutions. Indeed, if no new preservation procedures are developed, the major drawback in current protozoological techniques shall never be evaded. In addition, the alternative mounting media tested in this investigation are more economical than other commercial toluene-free alternatives such as Vectamount®. Moreover, these glues present low health hazard compared to toluene-based mounting media. However, precautionary measures must be taken into consideration through careful handling, since commercial adhesives are not totally harmless to the skin. Finally, it is important to note that the alternative preservation methods may be useful in education for medical and