Published in final edited form as:
Exp Parasitol. 2013 October ; 135(2): 257–261. doi:10.1016/j.exppara.2013.07.002.

Use of RNA*later*® as a preservation method for parasitic coprology studies in wild-living chimpanzees

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Abstract

We evaluated the use of an RNA stabilisation buffer, RNA*later*® (Ambion, Austin, Texas), as a preservation medium for parasitic coprology analysis of faecal samples collected from chimpanzees living in the wild (*Pan troglodytes troglodytes*). Thirty faecal samples collected in the forests of south-east Cameroon (Mambele area) from 2003 to 2011 were preserved in RNA*later*® at −80 °C and analysed for their parasite content. We identified and counted parasitic elements and assessed their shape, size and morphology in relation to the storage time of the samples. We found that parasite elements were identifiable in RNA*later*® preserved samples after as many as 7 years, showing that RNA*later*® could be an effective and reliable preservation medium for coprology. Thus, its use could be an interesting way to optimise sample collection for several types of studies (parasitology and bacteriology/virology) at once, especially considering the logistically challenging and time-consuming field campaigns needed to obtain these faecal samples.

Keywords

Wild-living chimpanzees; Endoparasites; Parasitic coprology; RNA*later*®

1. Introduction

In the tropical forest of the Congo basin, intensive logging, mining and bushmeat hunting have increased the contact between humans and non-human primates (NHPs). Such close contact may facilitate the zoonotic transmission of potentially pathogenic micro-organisms from NHPs to humans, with consequences for human health, as well as from humans to NHPs, with consequences for wildlife conservation (Calvignac-Spencer et al., 2012). It is therefore essential to implement sustained surveillance programs designed to detect cross-species transmission in order to prevent the potential spread of emerging infectious diseases in both NHP and human populations.

As a non-invasive method, analysis of faecal samples has proven to be a convenient means of assessing the prevalence of pathogens in NHP populations in the wild. The majority of

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Conflicts of interest
The authors declare no conflict of interest.

gastrointestinal parasites that infect apes, as well as viral, bacterial and protozoan agents infecting NHPs, can be monitored through faecal analysis (Kooriyama et al., 2012; Gillespie et al., 2010; Bezjian et al., 2008; Gillespie and Chapman 2008).

Analysing fresh stool samples in the field is challenging in many ways, so collected samples are usually preserved in a 10% formalin fixative solution and analysed several weeks or months later. The use of formalin, a formaldehyde containing solution, has several drawbacks. First, formaldehyde has a low vapour pressure point (35 °C), which may be inappropriate under tropical weather conditions. Secondly, it is a known skin, eye, and respiratory tract irritant and has been shown to be a carcinogen; therefore, its use should be limited to adequate lab facilities. Lastly, formaldehyde induces cross-linking and degradation of DNA, which is unsuitable if the samples are intended for molecular biology-based studies (Coombs et al., 1999; Williams et al., 1999).

To preserve nucleic acids (DNA or RNA) and to allow for molecular analyses on faecal samples collected in the wild from NHPs, other storage media have been used, including 70% or 96% ethanol (Nakamura et al., 2011; Uenishi et al., 2007), RNA*later*® (Ambion, Inc.) (Ochman et al., 2010; Szekely et al., 2010) and ethanol followed by desiccation using silica (Nsubuga et al., 2004). Freezing the samples in liquid nitrogen is also an effective means of preservation. However, when freezing was not possible, compared to the other methods of storage, RNA*later*® proved the most efficient method for preservation of microbial DNA (Vlčková et al., 2012; Nechvatal et al., 2008). RNA*later*® is an aqueous, non-toxic storage reagent that rapidly permeates tissues to stabilise and protect cellular RNA and DNA *in situ* in unfrozen specimens. RNA*later*® has been adopted by an increasing number of researchers focusing on NHPs viral infections (Etienne et al., 2012; Liu et al., 2010; Whittier et al., 2010; Locatelli et al., 2008; van heuverswyn et al., 2007; Keele et al., 2006; Santiago et al., 2003). It is also effective for preservation of *Plasmodium* DNA in faecal samples (Liu et al., 2010).

Considering the problems associated with storing faecal samples in a formalin solution for parasitic coprology, we decided to assess the feasibility of conducting microscopic examination of faecal samples stored in RNA*later*®. We selected and analysed chimpanzee faecal samples stored in RNA*later*® collected in the rainforest of southeast Cameroon between 2003 and 2011 in the context of a long-term study assessing the prevalence and genetic diversity of Simian Immunodeficiency Viruses (SIV).

2. Materials and methods

2.1. Sample collection and species identification

We randomly selected 30 faecal samples from the stool bank of the UMI 233 “TransVIHMI” laboratory in Montpellier. These samples were collected from chimpanzees (*Pan troglodytes troglodytes*) living in the forests of southeast Cameroon (Mambele area) between 2003 and 2011. They were stored in RNA*later*® at the time of collection then subsequently frozen at −80 °C one to three weeks later. The host species was confirmed by mtDNA analyses, as described previously (Keele et al., 2006; Van Heuverswyn et al., 2006; van der Kuyl et al., 1995). Briefly, a QIAamp stool DNA miniprep kit (Qiagen, Valencia, CA) was used to extract faecal DNA. Two millilitres of faecal sample were used to obtain a final elution volume of 100 μl of faecal DNA. A ~450- to 500-bp fragment spanning the hypervariable D-loop region was amplified using primers L15997 and H16498 and/or a 386-bp fragment spanning the 12S gene was amplified using primers 12S-L1091 and 12S-H1478. The sequences obtained using a 3130xl Genetic Analyser (Applied Biosystems, France) were aligned using the Seqman DNAStar (Lasergene, Madison, USA) software. The

species of animal providing the samples was confirmed by neighbour-joining analysis using the CLUSTAL X 2.0 program (Thompson et al., 1997)

2.2. Coprology

Once the species identification of the host was confirmed, we assessed the parasite content of the samples using the following method: for each sample, one millilitre of 50:50 stool:RNA*later*® mix was thawed at room temperature. Each sample was then vortexed briefly. One hundred microliters of this solution was mixed with 100 μl of physiological serum to avoid crystallisation or a multiple layer effect on the slides due to the high salt concentration in the RNA*later*® buffer. Then, a 50 μl aliquot of the sample-physiological serum mix was smeared on a slide and observed directly under a microscope (Olympus BX41). A second slide was prepared using Para-selles KOP Color II (Fumouze) dye (10 μl of dye for 50 μl of stool/physiological serum mix). When the results of the slides were both negative or a significant discrepancy was found between colored and not colored slides, either in the type or number of parasitic elements, two additional smears were performed. The species, number and shape of parasitic elements, as well as their ability to be dyed, were recorded. A possible effect of the date of collection on the results of the stool examination was considered. It should be noted that we tried concentration methods on the RNA*later*® samples (Bailenger method, Iodesin-Color, Para-selles KOP Color II Kit, Fumouze diagnostics, France) but they proved to be unsuccessful due to the high salt concentration of the RNA*later*® buffer.

To compare the efficiency of RNA*later*® and 10% formalin in preserving the parasitic elements, ten matched samples that were collected in 2011 and preserved in 10% formalin were analysed in parallel.

2.3. Molecular biology assay

To show the utility of RNA*later*® versus formalin samples in combined coprology-molecular biology studies, a PCR detection assay for *Blastocystis* sp. was performed on DNA extracted from ten RNA*later*® and 10 matched formalin samples collected in 2011. *Blastocytis* was chosen for this test because of the difficulties in detecting it by direct microscopy, especially in stool samples rich in plant debris. Briefly, a QIAamp stool DNA miniprep kit (Qiagen, Valencia, CA) was used to extract faecal DNA from each sample under the conditions specified by the manufacturer for parasites. Two milliliters of faecal sample were used to obtain a final elution volume of 100 μl of faecal DNA. PCR was performed as described by Grabensteiner and Hess, 2006 with the following conditions: forward primer BLF 5′-TAACCGTAGTAATTCTAGGGC-3′, reverse primer BLR 5′-AACGTTAATATACGCTATTGG-3′, denaturation at 94 °C for 5 min, 40 cycles of 94 °C for 1 min, 53 °C for 1 min, and 72 °C for 1 min, followed by a final extension step of 72 °C for 10 min. PCR products were separated on a 1% agarose-ethidium bromide gel and visualised under UV. Positive samples were sent to Beckman Coulter Genomics UK for sequencing.

3. Results

Parasitic coprology performed on our RNA*later*® sample set allowed us to detect parasitic elements (helminth eggs and protozoa cysts) in faeces that were several years old.

These analyses showed that samples collected in 2003 contained few identifiable parasitic elements, and those that were observed had degraded morphological aspects (Table 1). Samples from 2005 to 2011 were less degraded, and we could identify the parasitological content more easily (Table 1). In this sample set, we were able to observe the presence of

several protozoan cysts belonging to the *Endolimax* and *Entamoeba* genera (with diameters ranging from 6–8 μm to 16–20 μm, so possibly from different species) as well as *Blastocystis* cells (Table 1 and Fig. 1A–D).

Moreover, we detected *Trichuris* eggs, *Enterobius* eggs and strongylid eggs of different sizes, which likely represent different genera (Table 1 and Fig. 1E–G). We also observed eggs of an undetermined helminth species (Fig. 1 H) and were able to detect *Troglodytella* trophozoites (Table 1 and Fig. 1I).

The types of parasitic elements observed in the matched samples (RNA*later*® *vs*. 10% formalin) were similar in both sets of samples (Table 1: Chimp-21–30 *vs*. Chimp-21*–30*, respectively). However, parasites preserved in RNA*later*® displayed more degraded cellular content (retracted cytoplasm for the protozoa cysts and lysed blastomere cells for the helminth eggs; Fig. 1A–H) than those from the formalin preserved samples, with the cytoplasmic content being better preserved in formalin (Fig. 1J–O). The increased degradation of the parasitic content in the RNA*later*® buffer may be due to the high salt hyper-osmotic content of this medium combined with storage at −80 °C. Thus, we were able to determine the genera of well-described parasites (helminth eggs, amoeba cysts) stored in RNA*later*® but this induced degradation was a limiting factor for species identification relying on fine internal structures (number of nuclei, position of the chromatin for the *Entamoeba* species, number of blastomeres for *Ancylostoma duodenale* etc.). The detection and identification of smaller elements (*Endolimax* and *Iodamoeba* cysts) was also more difficult in the samples preserved in RNA*later*® than in 10% formalin. Finally, as the high salt concentration of RNA*later*® buffer modifies the density of the sample, faecal floatation and sedimentation concentration methods were inappropriate. As such, the samples were observable only by direct smear as described above.

With respect to molecular detection assays for parasite elements (*Blastocystis* sp.), as expected, none of the formalin-preserved samples (Chimp21*–30* gave a positive result, whereas the same matched samples preserved in RNA*later*® were all positive by PCR (Fig. 2). Sequencing of products and sequence analyses by BLAST confirmed that 8 of these PCR fragments (Chimp-23–30) indeed matched *Blastocystis sp* (Blastocystis subtype 1 Chimp) whereas the sequences obtained from the Chimp-21 and Chimp-22 individuals matched undetermined uncultured alveolate eukaryotes (data not shown).

4. Discussion

In this study, we observed that RNA*later*® has some minor limitations when used as a preservation method for parasite coprology. In fact, we could not obtain any data using concentration methods because of the high salinity of the buffer. As a consequence, we were only able to rely on direct smears for observation. Moreover, RNA*later*® is less efficient than formalin for the preservation of fine internal structures of some fragile parasite eggs or cysts.

Nevertheless, we were able to identify *Endolimax* cysts, *Entamoeba* cysts, *Troglodytella* trophozoites, *Blastocystis* cells, *Trichuris* eggs, *Enterobius* eggs, strongylid eggs of different sizes (possibly *Trichostrongylus, Oesophagostomum, Strongyloides* and *Ancylostoma*), and several nematode larvae (not shown), as well as eggs from an unknown helminth in RNA*later*®-preserved samples. The fact that we were able to observe nematode eggs, ranging from resistant (*Trichuris* eggs, Sanguinetti et al., 2005) to more fragile (strongylid eggs, Waruiru et al., 1998), supports the efficacy of RNA*later*® as a preservation method. The observation of *Troglodytella* was also particularly interesting, demonstrating that it is possible to identify a ciliate trophozoite (which is a more fragile cellular element compared

to parasite cysts or thick-walled helminth eggs) in samples stored in RNA*later*®. Moreover, we were able to identify these parasites in samples aged up to 7 years old that had been kept at 80 °C and which, in some cases, were already thawed and frozen several times during previous studies. Given our results, RNA*later*® stool samples stored for up to seven years in laboratory banks worldwide could therefore represent an interesting source for retrospective studies on gastrointestinal parasites of endangered animals. Finally, a probe molecular assay showed that combined molecular biology studies (detection of *Blastocystis sp*. by PCR in this article) and parasitic coprology studies can be performed on RNA*later*® samples in parallel, something that is impossible with formalin-preserved samples.

Until now, NHP samples stored in RNA*later*® were only used for molecular biology analyses such as species identification, genotyping, and viral/bacterial/fungal molecular diagnoses. We show here for the first time that they can also be used to measure in parallel the extent and diversity of gastrointestinal endoparasites, thereby providing a broader spectrum of information regarding the pathogens infecting rare or endangered NHP species.

Finally, these results showed that, in the future, faecal sample collection in the field can be simplified by working with a single preservation buffer, thus avoiding issues related to the use of formaldehyde.

Acknowledgments

We thank the staff and the SIV team of Innocent Ndong Bass, Aimé Mebenga and Joseph Moudindo from PRESICA for logistical support in Cameroon and the Cameroonian Ministries of Health, Environment and Forestry, and Research for permission to collect samples in Cameroon. We also thank Dr L. Mondolot, Pr. Y. Pelissier and Dr. S. Bertout for their useful help with the identification of false positives.

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HIGHLIGHTS

- We evaluated if RNA*later*® could preserve parasites in wild-living chimpanzees stools.
- RNA*later*® is suited for samples collections in logistically challenging areas.
- Parasites were identifiable in samples preserved at −80 °C for as many as 7 years.
- RNA*later*® may facilitate combined studies (molecular analyses and coprology together).

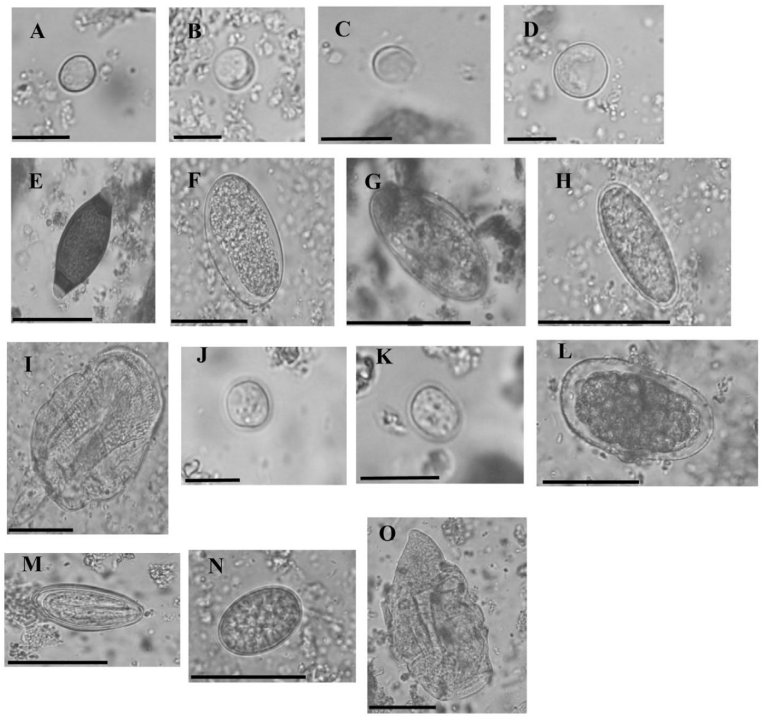

Fig. 1.

Parasite elements observed by microscopy in stools collected from wild chimpanzees. Elements in A to I were found in chimpanzee stools collected in Cameroon from 2003 to 2011 and preserved in RNAlater buffer at −80 °C. Elements in J–O were found in samples collected in the same area in 2011 and preserved in 10% formaldehyde buffer at 4 °C. A: *Endolimax cyst*. B: small unidentified amoeba cyst (*Entamoeba histolytica/E. hartmanni* ?) C: *Blastocystis* cell. D: *Entamoeba* cyst (*Entamoeba histolytica/ E.coli* ?) E: *Trichuris* egg. F: Strongylid egg. G:*Enterobius* egg. H: unidentified helminth egg. I: *Troglodytella* trophozoite. J: *Iodamoeba* cyst. K: *Endolimax* cyst. L: Strongylid egg. M: *Enterobius* egg. N: unidentified helminth egg. O: *Troglodytella* trophozoite. Scale bars indicate 10 μm for elements A–D, J, and K and 50 μm for elements E–I, L–O.

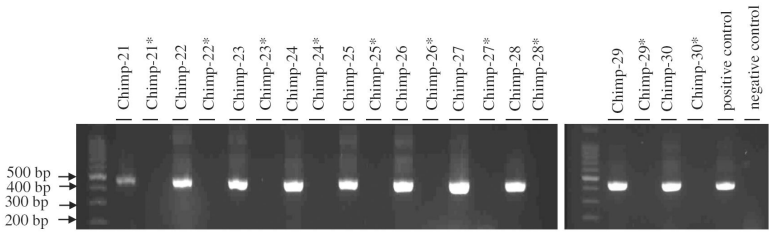

Fig. 2.

Blastocystis sp. PCR detection assay. Detection of *Blastocystis sp* was performed by PCR on total DNA extracted from chimpanzee stools with specific *Blastocystis* primers. Chimp-21–30 correspond to PCR profiles obtained with DNA extracted from stool samples preserved in RNA*later*® Chimp-21*–30* correspond to PCR profiles obtained with DNA extracted from samples obtained from the same animals as Chimp-21–30 but preserved in formalin. The positive control PCR profile was obtained using DNA extracted from a stool sample tested positive for *Blastocystis* sp. by direct microscopy that had been discarded from the study because the shedding species was not chimpanzee but *Gorilla gorilla*. The negative control PCR profile was obtained using water instead of DNA.

Table 1

Type and number of parasite elements found in samples collected from 2003 to 2011 in the Mambele area of Cameroon.

Sample	Year of collection	Method of preservation	Observed elements, helminths	Observed elements, protozoas
Chimp-1	2003	RNA*later*®	4–5 larvae	Small amoeba cysts and *Blastocystis*
Chimp-2	2003	RNA*later*®	1 larvae, 1 *Trichuris* egg	0
Chimp-3	2003	RNA*later*®	1 larvae	1 *Troglodytella* trophozoite
Chimp-4	2003	RNA*later*®	0	0
Chimp-5	2003	RNA*later*®	0	0
Chimp-6	2005	RNA*later*®	2 strongylid eggs	*Endolimax* cysts
Chimp-7	2005	RNA*later*®	1 strongylid egg, 4–6 unknown eggs	0
Chimp-8	2005	RNA*later*®	0	>10 *Troglodytella*
Chimp-9	2005	RNA*later*®	3–4 strongylid eggs	0
Chimp-10	2005	RNA*later*®	2 strongylid eggs	>30 *Troglodytella*
Chimp-11	2007	RNA*later*®	2–3 strongylid eggs	0
Chimp-12	2007	RNA*later*®	10–12 strongylid eggs	0
Chimp-13	2007	RNA*later*®	3–4 strongylid eggs	0
Chimp-14	2007	RNA*later*®	4–5 strongylid eggs	*Blastocystis*
Chimp-15	2007	RNA*later*®	6–10 strongylid eggs	*Blastocystis*
Chimp-16	2010	RNA*later*®	3–8 strongylid eggs, 1 *Enterobius* egg	*Endolimax*, 4–5 *Entamoeba* cysts, *Blastocystis*
Chimp-17	2010	RNA*later*®	0	>10 *Troglodytella*
Chimp-18	2010	RNA*later*®	4–5 strongylid eggs	1–2 *Troglodytella*
Chimp-19	2010	RNA*later*®	1 strongylid egg	*2 Troglodytella*
Chimp-20	2010	RNA*later*®	2 strongylid eggs	0
Chimp-21	2011	RNA*later*®	1 larvae	0
Chimp-22	2011	RNA*later*®	12 larva, 2–3 strongylid eggs	small amoebas cysts
Chimp-23	2011	RNA*later*®	1–2 strongylid eggs	0
Chimp-24	2011	RNA*later*®	2 strongylid eggs	4–5 *Troglodytella*
Chimp-25	2011	RNA*later*®	2–3 strongylid eggs	>30 *Troglodytella*
Chimp-26	2011	RNA*later*®	1 larvae, 1 strongylid egg	0
Chimp-27	2011	RNA*later*®	2–3 strongylid eggs	7–10 *Troglodytellas*
Chimp-28	2011	RNA*later*®	1 larvae, 3 strongylid eggs	5 *Troglodytella*
Chimp-29	2011	RNA*later*®	3 strongylid eggs, unidentified eggs (+)	14 *Troglodytella*
Chimp-30	2011	RNA*later*®	2 strongylid eggs, unidentified eggs (+)	5 *Troglodytella*
Chimp-21*	2011	Formaline	0	*Blastocystis*
Chimp-22*	2011	Formaline	11 larva, 6–7 strongylid eggs	*Endolimax*
Chimp-23*	2011	Formaline	2 strongylid eggs, 1 *Enterobius*, egg	*Iodamoeba* cysts
Chimp-24*	2011	Formaline	0	11 *Troglodytella, Endolimax, Iodamoeba*

Sample	Year of collection	Method of preservation	Observed elements, helminths	Observed elements, protozoas
Chimp-25*	2011	Formaline	1–2 strongylid eggs	>30 *Troglodytella, Endolimax, lodamoeba*
Chimp-26*	2011	Formaline	2–3 strongylid egg	7–9 *Troglodytella*
Chimp-27*	2011	Formaline	4 strongylid eggs	5 *Troglodytella*
Chimp-28*	2011	Formaline	1 strongylid eggs	*4 Troglodytella*
Chimp-29*	2011	Formaline	2 strongylid eggs, unidentified eggs (+)	>20 *Troglodytella*
Chimp-30*	2011	Formaline	1 strongylid eggs, unidentified eggs (+)	5 *Troglodytella*

The samples numbered from Chimp-1 to Chimp-30 are samples preserved in RNA*later* at −80 °C.

The samples numbered Chimp-21–30* are the same samples as Chimp-21–30 but preserved in 10% formalin solution and at 4 °C.

The number of parasite elements was counted for each sample and is shown in the table.

The + sign indicates a very high density of unidentified helminth eggs on the slide (2–3 elements on average per microscope field at 40×).