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TECHNICAL NOTE

A new method for the sampling and preservation of placental specimens in low-resource settings for the identification of *P. falciparum* and analysis of nucleic acids

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ABSTRACT

Collection, preservation, and shipment of histological specimens in low-resource settings is challenging. We present a novel method that achieved excellent preservation of placental specimens from rural Mali by using formalin fixation, ethanol dehydration, and long-term storage in a solar-powered freezer. Sample preservation success was 92%, permitting evaluation of current and past malaria infection, anemia, placental maturity, and inflammation. Using RNAscope® hybridization we were able to visualize cell-specific gene expression patterns in the formalin-fixed paraffin-embedded (FFPE) specimens. Additionally, our method entailed mirrored sampling from the two cut faces of a cotyledon, one for the FFPE workflows and the other for storage in RNAlater™ and RNA-seq.

KEYWORDS

Field-work; placenta; histology; RNAscope®; RNA-seq; malaria

Introduction

Neutral-buffered formalin is routinely used for tissue preservation including for the placenta [1–5]. For this project, we sought to preserve placentas to detect malaria infection. Existing limitations of neutral-buffered formalin include the introduction of fixation artifacts that are known to develop under conditions of prolonged fixation, elevated temperature, and high humidity [6]. These artifacts can resemble hemozoin deposits, the waste product of the malaria parasite, particularly when acidic formalin is used [7]. We collected placental specimens in a rural location in Mali, West Africa, that is endemic for malaria (*Plasmodium falciparum*) and where ambient temperature often exceeds 40°C. We report a new collection protocol for placental specimens collected following delivery that minimized the formalin artifacts, required little on site infrastructure, and resulted in specimens that could be shipped on dry ice. Our collection and preservation protocol routinely produced high quality histological specimens suitable for the assessment of morphological features of the delivered placenta, including changes associated with active or past placental malaria infection, and suitable for *in situ* RNA hybridization. In parallel, we also collected specimens in RNAlater™ without fixation for molecular analysis of nucleic acids.

Methods

Collection and processing

The placentas came from women who participated in a longitudinal study on the Bandiagara Escarpment in Mali. The cohort and IRB approval is described in detail in Vincenz et al. [8,9]. To obtain specimens from term placentas, we identified well-formed cotyledons within the central two-thirds of the maternal surface of the placenta. We then cut each cotyledon in half and dissected the histological specimen from the interior of one cut face and the specimen for nucleic acid analysis from the opposite face. The areas sampled consisted mainly of fetal villi and maternal intervillous space. We performed this mirrored sampling on two cotyledons per placenta generating 638 histological and nucleic acid specimens.

Histological specimens were placed in tissue cassettes and fixed with formaldehyde (Merck, purchased in Bamako, the capital city of Mali) freshly diluted 1:10 with phosphate buffered saline (PBS) to 3.7% final concentration. PBS was reconstituted using PBS tablets (Sigma-Aldrich, USA) and distilled water. Fixation was for 36 hr on ice followed by a 30 min wash in 70% ethanol. The tissue cassettes were shaken off, transferred to plastic bags, and stored in a solar freezer (~ −20°C) for up to 18 months prior to shipment on dry ice via World Courier to a −80°C freezer at the University of Michigan.

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Specimen collection and nucleic acid purification for RNA-seq were described previously [8]. RNA quality of these samples and targeted RNA-seq results were presented in Wu et al. [10].

Slide preparation and visualization

The tissue cassettes were removed from the −80°C freezer within a year of their arrival and submerged at room temperature in 70% ethanol prior to processing at the University of Michigan Tissue and Molecular Pathology (TMP) core. Tissue blocks were embedded in paraffin and two, 4 µm thick sections were cut from each sample and stained with hematoxylin and eosin (H&E) or Giemsa. Histology slides were examined under an Olympus BX40 microscope (Olympus Lifescience, USA). Polarized light along with location of pigments permitted hemozoin to be distinguished from formaldehyde artifacts [11]. The RNAscope® Multiplex Fluorescent V2 assay (Advanced Cell Diagnostics, USA) was performed according to the user manual using unstained specimens (Figure 1) [12]. The total cost for the RNAscope kit and four fluorophores (only three were used) totaled $5031.20. The selected RNA probes are implicated in the establishment of imprinting domains [13–15]. *KCNQ1OT1* (Cat. # 410631), *MEG3* (Cat # 400821-C2), and *ERVW-1* (Cat. # 536811-C3) RNA probes were used in the assay. Opal 570 (FP1488001KT), Opal 620 (FP1495001KT), and Opal 690 (FP1497001KT) (Akoya Biosciences) were used as compatible fluorophores. The method was replicated from a previous study except that our DAPI incubation time was 5 min instead of 30 sec [16]. Slides with FFPE sections were dried at 60°C for one hour, then dehydrated in increasing concentrations of ethanol, cleared in xylenes, and rehydrated in decreasing concentrations of ethanol. Endogenous peroxidase activity was blocked with H_2O_2 for 10 min. Slides were then boiled for 15 min in RNAscope target retrieval agent, incubated in RT 100% ethanol for 3 min, and dried at 60°C for 5 minutes. A hydrophobic barrier was drawn around the sections and RNAscope Protease Plus was then applied on slides and incubated for 30 min at 40°C. Hybridization was performed by applying target probes to sections for 2 h at 40°C. One positive (human sequences of: *UBC, POLR2A, PPIB*) and one negative hybridization (bacterial gene: *dapB*) was included in each batch of slides. Amplification for each probe was performed according to the protocol for the RNAscope Multiplex Fluorescent v2 assay. Slides were counterstained with DAPI, incubated at RT for 5 min, and mounted with ProLong Gold (Thermo Fisher Scientific, USA). Confocal images were acquired with a Leica Stellaris 5 confocal microscope (Leica

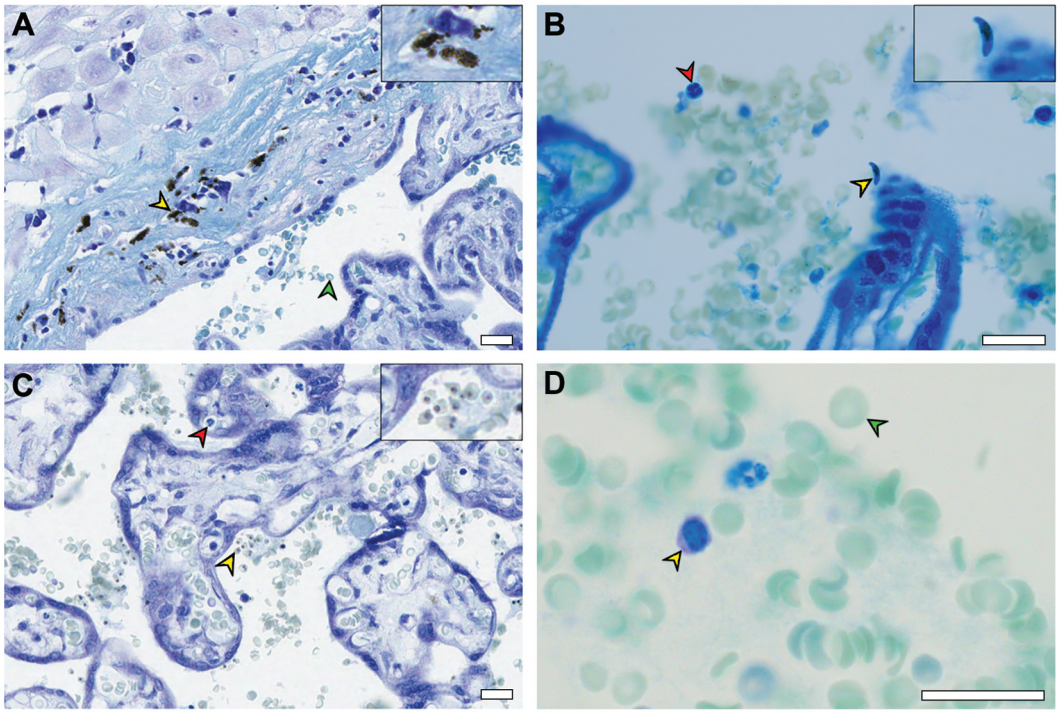

Figure 1. Representative micrographs from Giemsa-stained sections of malaria-infected term placentas collected in the field using our novel protocol. Yellow arrows point to features of interest, red arrows point to neutrophils, and. green arrows point to individual erythrocytes. Insets show enlarged features of interest in A, B, and C. (A) shows hemozoin, (B) shows gametocyte form, (C) shows trophozoite form, (D) shows schizont form. Scale bars = 20 µm.

Microsystems, USA) using the 63x objective (NA 1.40) under Type F immersion oil for a total magnification of 630x.

We scored histological preservation as 0 for samples without fixation issues, 1 for samples with damage limited to erythrocytes but still readable, and 2 if the samples were completely unreadable.

Results & discussion

The Giemsa stained specimens (Figure 1) show intact placental morphology, with excellent preservation of the villi, macrophages (not pictured), neutrophils, and erythrocytes. Figure 1A shows brown hemozoin, the waste product of *P. falciparum*, located in perivillous fibrin of the placenta. Figure 1B shows the gametocyte stage of *P. falciparum* [17], which is the sexual form. Figure 1C shows a severe case of malaria with many *P. falciparum* trophozoites (activated feeding stage). Figure 1D shows a schizont, a mature stage of *P. falciparum*. The asexual stage parasites in A, C, and D indicate active infections. Figure 1B demonstrates the sexual erythrocytic stage of the parasite that is ingested by mosquitoes during a blood meal [17]. The presence of only hemozoin indicates past infection.

RNAscope® in-situ hybridization was performed to examine whether the preservation of RNA was sufficient to visualize the expression of specific genes (Figure 2). The RNAscope® probes were for two imprinted lncRNAs, *KCNQ1OT1* and *MEG3*, and a protein-coding RNA, *ERVW-1*, which is a marker for trophoblasts. All three probes generated strong signals in different cells indicative of specific hybridization. Thus, the specimens collected with this protocol were of sufficient quality to provide spatial information on the expression of specific genes.

We collected 638 histology samples from 322 placentas. We excluded 49 due to sample properties unrelated to fixation (e.g. ischemia, bad tissue sectioning). Of the remaining 589 samples, 544 (92%) samples were readable (score of 0 or 1). 517 samples were scored a 0, while 27 samples were scored a 1. The 45 unreadable specimens (scored as a 2) could not be assessed for malaria infection due to loss of structure reminiscent of freezing artifacts. We did not attempt to use 30% sucrose as a cryoprotectant due to the inability to assess osmolarity artifacts in the field. Freezer time was calculated for each specimen and defined as the days between the birth of the placenta and the shipment arrival in the USA. A two-sample t-test was performed to test if unreadable specimens spent more time in the freezer on average when compared with

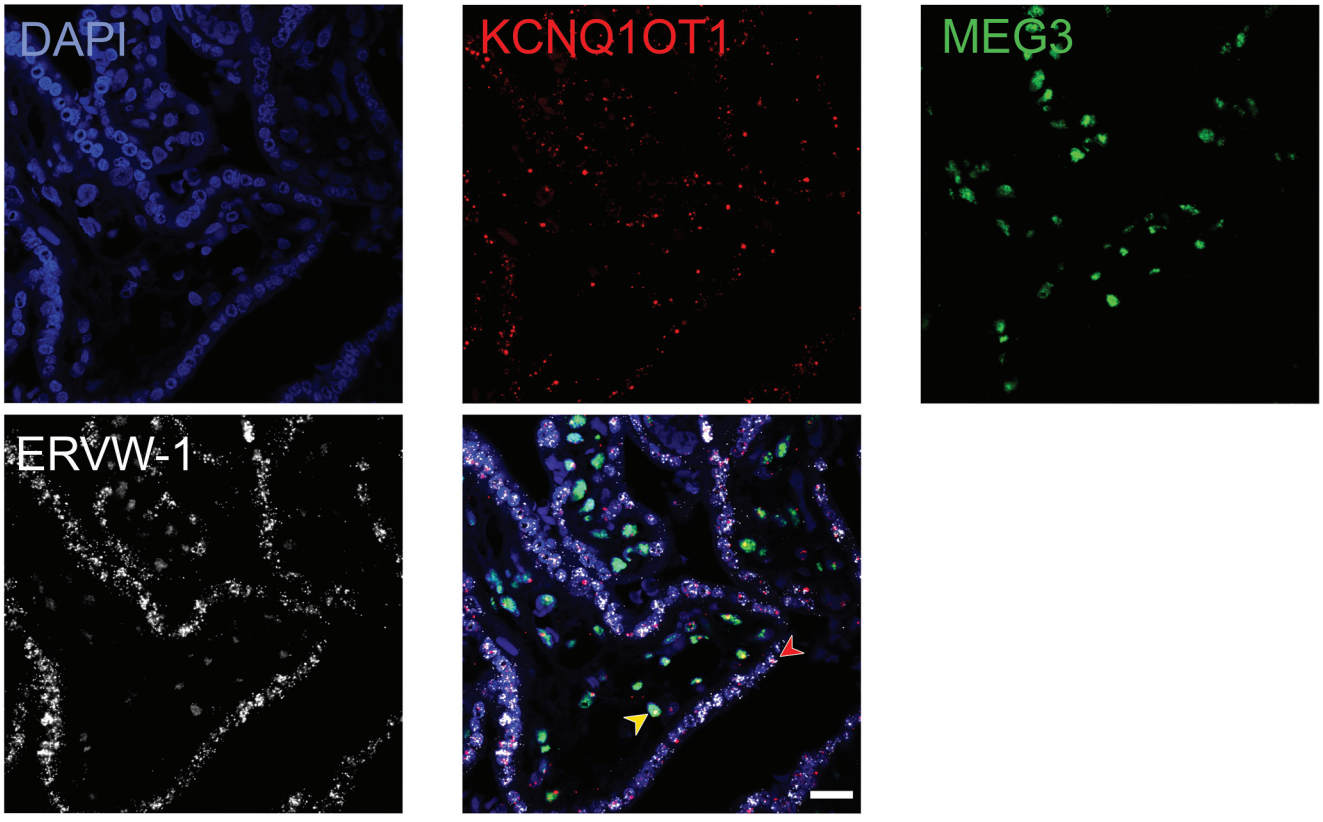

Figure 2. RNAScope fluorescent micrographs of DAPI for nuclei, *KCNQ1OT1, MEG3, ERVW-1*, and a merged micrograph of all four where a red arrow points to a line of syncytiotrophoblast surrounding the fetal villous. The yellow arrow points to a mesenchymal cell located within the fetal villous. Scale bar =20 µm.

readable specimens. The test found t = −1.95, df = 47.7, p-value = 0.06. These results imply that the unreadable samples generally spent more time in the freezers at the collection sites, although this was mostly characteristic of one group of samples and not all samples (Supplemental Figure 1). Thus, we recommend limiting freezer time (−20°C), but also recognize that frequent shipping is often not feasible due to cost and availability of dry ice.

Conclusions

The protocol presented here will improve the collection of placental specimens in low resource settings. Formaldehyde is widely available, but due to its toxicity, appropriate training of personnel is essential. Solar freezers are available in most urban areas and can be installed in rural settings. Maintenance costs consist mainly in the periodic exchange of lead acid batteries. The overall quality of the FFPE specimens was excellent and permitted the evaluation of current and past malaria infection, anemia, placental maturity, and inflammation, as well as visualization of gene expression using RNAscope®. The RNA specimens produced high quality data using targeted RNA-seq, as shown in Wu et al. [10].

Disclosure statement

No potential conflict of interest was reported by the author(s).

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