Evaluation of a Single Vial/Fixative for Parasitic Testing by Microscopy, Antigen Detection, and Real-Time PCR

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Introduction

Stool collection kits for parasites historically contained three tubes - 10% formalin, PVA (Poly-vinyl Alcohol), and a clean tube. With these three tubes, a variety of tests can be performed: Ova & parasite exams (O&P), specialty stains, antigen detection, and molecular assays. Due to environmental and health hazards related to formalin and mercuric PVA, formalin-free fixatives have been developed that allow parasite detection to be performed from a single vial. We compared our current method of 10% formalin/PVA collection to the use of a single vial mini Parasep® SF tube with either Alcorfix® or SafEFix® (Apacor, Berkshire, UK) for O&P, antigen, and molecular testing.

Methods

Spin-Con Methodology with Formalin and PVA:

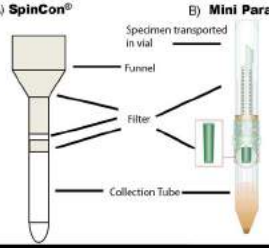
Stool is submitted to the lab in formalin and PVA vials. Each vial is manually poured-off into a SpinCon® funnel attached to filters and a collection tube (Figure 1A). Tubes are centrifuged for 10 mins. The supernatent is poured-off leaving a pellet which is then used to make either Trichrome (PVA) or Wet-mount (Formalin) slides. Alternatively, Modified acid-fast or modified trichrome stains can be made from the formalin pellet.

Parasep Methodology with Alcorfix®:

The device is provided to the patient as two separate components which are assembled after collection/inoculation for subsequent transport and processing (Figure 1B). The fixative is contained in a flat-bottom tube containing a screw-off cap, while the vertical filtration device is attached to the conical collection tube assembly. Patients collect two level spoons of stool which are added to the Alcorfix®- or SafEFix®-containing portion of the tube. Alcorfix® is an alcohol-based fixative (ethanol, PVA, isopropanol, methyl alcohol, acetic acid, glycerin, and zinc sulfate) and SafEFix® is a proprietary fixative that is environmentally friendly and alcohol free. Tubes received in the lab are briefly mixed and centrifuged at 400 *xg* for 2 mins. The supernatant is poured-off leaving a pellet which is then used to make Trichrome and Wet-mount slides.

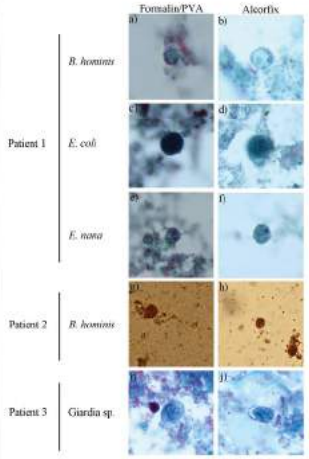
FIGURE 1: Diagram of SpinCon® & Mini Parasep® SF concentrators

A) **SpinCon®** B) **Mini Parasep® SF**



Results

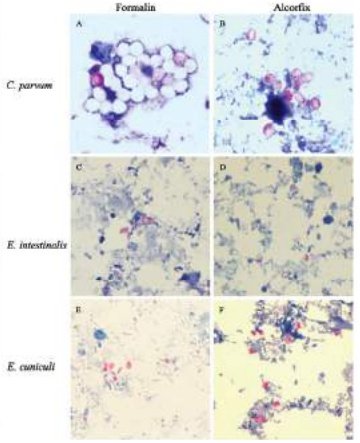
FIGURE 2: Clinical evaluation of Alcorfix® by prospective co-collection study



The ability of Alcorfix® to preserve morphology and serve as a suitable alternative to PVA and formalin was evaluated in real-time at the University of Utah hospital and clinics. Stool collection kits with the standard PVA /formalin and the Mini Parasep® tube containing Alcorfix® were provided for O&P collection. A total of 26 specimens were submitted for testing in which both fixatives were filled with stool at the required specimen volumes. Three specimens contained parasites of the 26 submissions meeting inclusion criteria (Figure 2). Mini Parasep® tubes containing Alcorfix® showed acceptable performance for conventional trichrome stain and wet mount evaluation; however no coccidian parasites were encountered in our prospective co-collection study.

Results

FIGURE 3: Alcorfix compatibility with modified acid fast & modified trichrome stains



Stool spiked with viable *C. parvum* or microsporidia were separated into vials containing formalin and Alcorfix® and prepared for microscopic examination with a permanent acid-fast stain after concentration in Mini Parasep® tubes. The morphology of the oocysts was maintained in both fixatives (Figure 3A-B). To ensure Alcorfix® with Mini Parasep® tubes can detect microsporidia, live spores were procured, spiked into stool, and fixed in both 10% formalin and Alcorfix® separately. Modified trichrome staining was performed on both fixatives and evaluated microscopically. Both preparations showed conserved morphology predictable for microsporidia, as well as adequate stain retention for both *E. intestinalis* and *E. cuniculi* (Figure 3C-F).

Results

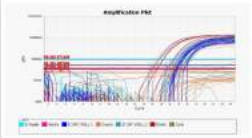
FIGURE 5: Workflow analysis between the two types of stool concentrators

Three laboratory technicians independently processed batches of 30 O&Ps prepared in PVA/formalin tubes and Mini Parasep® tubes. Each step was timed and recorded, and the average and standard deviation was calculated for each batch and concentration method. The average time to prepare a run of 30 O&P specimens was 52 minutes (4:25 standard deviation) using SpinCon® concentration for PVA/formalin, versus 19:45 (standard deviation 1:52) for Mini Parasep®. This difference in workflow represents an average time savings of 32:01, with less variability in total time for processing.

FIGURE 4: Alcorfix® and SafEFix® can be used for molecular applications and

A)

	Alcorfix #1	Alcorfix #2	SafEFix #1	SafEFix #2	Formalin #1	Formalin #2	Original CT	Organism
R & D A116D	26.1	25.9	27.1	26.1	29.4	29.1	27.9	E. histo
R & D A119D	27.1	26.1	26.3	27.0	33.4	32.9	30.3	E. histo
R & D A117D	25.7	25.4	27.1	26.3	33.0	30.9	25.2	E. histo
R & D A114D	29.3	28.4	30.6	28.7	31.8	31.4	29.6	E. histo
R & D A929D	31.4	31.6	ND	ND	ND	ND	32.2	Crypto



B)

	#1	Replicate1 1:100 dilution	Replicate2 1:100 dilution	2 week Stability
Formalin	1.38	0.37	0.39	1.25
Alcorfix	1.08	0.74	0.82	0.3
SafEFix	1.91	0.74	0.75	1.54

A) **Real-Time PCR Applications**

Stool positive for *Entamoeba histolytica* or *Cryptosporidium spp.* were fixed in Alcorfix®, SafEFix®, or Formalin at a 1:3 ratio respectively and stored for three days. Nucleic acid was extracted from the fixed specimens and assayed on a laboratory developed, parasitic real-time PCR assay. Specimens were extracted in duplicate and run on separate days.

B) **ELISA Applications**

Cryptosporidium parvum was spiked into stool and fixed with Alcorfix®, Formalin, or SafEFix® at a 1:3 ratio respectively. Specimens were run on the TechLab® *Cryptosporidium* II ELISA. Diluted specimens were tested and stability was examined up to 2 weeks. All fixatives were compatible and were stable for 1 week. Antigen appeared to degrade between 1-2 weeks, as week 2 tetsing showed a decrease in signal for Alcorfix®

Conclusions

- An additional comparative study performed in our laboratory using both concentration devices on 47 known-positive stool samples revealed:
  - Overall equivalent performance between both concentration methods
  - One discrepant result: *B. hominis* and *Entamoeba coli* detected by Parasep® concentration whereas SpinCon® only allowed detection of *B. hominis*.
- Prospective co-collection shows performance of Alcorfix® is equivalent to PVA/formalin for trichrome stain and wetmount preparations
- Spiking of stool with *C. parvum* or microsporidia and fixing with Alcorfix® or Formalin showed equal or better performance for Alcorfix®
  - SafEFix® also shows equal performance with *C. parvum* (DNS) staining (Microsporidia studies will be conducted in the future)
- Alcorfix and SafEFix® are compatible for molecular applications and ELISA assays
  - Alcorfix may show a slight decrease in antigen stability past 1 week; further studies are needed to confirm this observation
- The single-vial Parasep® concentrator along with Alcorfix® was superior in a work flow analysis saving over 30 mins of time per 30 sample run.

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