JCM Accepted Manuscript Posted Online 27 May 2015
J. Clin. Microbiol. doi:10.1128/JCM.00838-15

Clinical and analytical evaluation of a single vial stool collection device with formalin free fixative for improved processing and comprehensive detection of gastrointestinal parasites.

Brianne A. Couturier[1], Ryan Jensen[1], Nora Arias[1], Michael Heffron[1], Elyse Gubler[1], Kristin Case[1], Jason Gowans[1], and Marc Roger Couturier[1,2]#

ARUP Laboratories, Institute for Clinical and Experimental Pathology, Salt Lake City, Utah, USA[1]; Department of Pathology, University of Utah School of Medicine, Salt Lake City, Utah, USA[2]

Running head: Improved ova and parasite specimen processing

Address correspondence to Marc Roger Couturier, marc.couturier@aruplab.com

Abstract:

Microscropic examination of feces is a standard laboratory method for diagnosing gastrointestinal parasite infections. In North America, the ova and parasite (O&P) examination is typically performed using stool that is chemically fixed in polyvinyl alcohol (PVA) and formalin, after which the stool is concentrated by filtration to enhance sensitivity. Mini Parasep® SF tubes allow for collection and concentration within a single collection vial. The goal of the study was to determine whether consolidated processing and concentration with the Parasep® tubes using an alcohol-based fixative (Alcorfix®) provides equivalent or better O&P examinations than processing of PVA/formalin-fixed stool using SpinCon® concentration. Parasep® tubes revealed equivalent filtration performance versus SpinCon® using PVA/formalin-fixed stool containing protozoa. Specimens co-collected in PVA/formalin and Alcorfix® in Parasep® tubes revealed comparable morphology and staining for various protozoa. Alcorfix® was effectively fixed live *Cryptosporidium* and Microsporidia such that morphology and staining was conserved for modified acid-fast and modified trichrome stains. A workflow analysis revealed significant time savings for batches of 10 or 30 O&P specimens compared to the same number of specimens in PVA/formalin tubes. Direct hands-on time savings with mini Parasep® tubes were 17:41 and 32:01 minutes for batches of 10 or 30 respectively. Parasep® tubes containing Alcorfix® provide significant workflow advantages to laboratories that process medium to high volumes of O&P specimens by streamlining processing and converting to a single tube. These improvements in workflow, reduction of formalin in the laboratory, and equivalent microscopy results are attractive advancements in O&P testing for North American diagnostic parasitology laboratories.

INTRODUCTION

Diagnosing gastrointestinal protozoa by microscopic examination is a well described laboratory technique that lends itself to decades of standardization and international adoption. While different institutions may adopt slightly different procedures for examining stools for ova and parasites (O&P), the core process in North America is largely conserved to: specimen fixation/collection, concentration, and microscopic evaluation by permanent-stained smear (typically trichrome, modified trichrome, or modified acid-fast) and wet mount evaluation. A significant advancement in the field of parasitology was made when single vial, alcohol-based fixatives were commercially produced to replace conventional formalin-based fixatives such as 10% buffered formalin or sodium acetate formalin [reviewed in Mc Hardy *et al.* (5)]. Formalin carries significant health hazards for both the patient and the laboratory worker, and also is environmentally and fiscally detrimental in terms of the impact of proper disposal. Many institutions have since made a conscious switch away from formalin to satisfy institutional or local government mandates to limit or eliminate the use of formalin in patient specimen collection and laboratory processes.

Several commercial fixatives exist that allow single-vial fixatives to accomplish both a permanent stained trichrome smear as well as a wet-mount preparation, and they have been described in multiple previous investigations (1, 4, 6). In spite of these beneficial advances in fixatives, a significant area of stagnation in fecal parasite testing has been in the collection and processing of fecal specimens for microscopic examination. Processing specimens for ova and parasite investigation is a largely manual process which involves chemical precipitation or filtration to achieve parasite concentration. In the case of filtration, which is the current standard in North American laboratories, manual transfer of stool from the original collection tube to the appropriate concentration tube is necessary (3). Concentrated specimens are then visualized microscopically using any combination of modalities, including wet mount

Journal of Clinical Microbiology

(with or without contrasting agent), trichrome stain, modified acid-fast stain, and modified trichrome stain using chromotrope 2R.

We performed a thorough analytical and clinical evaluation of a single vial, formalin-free fixative, AlcorFix®, and Mini Parasep® SF (solvent free) collection tube (Parasep®) combination from Apacor (Berkshire, England, UK). The Parasep® tube is a vertical filtration design that uses two separate vertical filtration steps (housed within the stool "spoon") to concentrate stool specimens without the need for volatile solvents such as ether or ethyl acetate. The device is provided to the patient as two separate components which are assembled after collection/inoculation for subsequent transport and processing (Figure 1). 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 (Figure 1). Patients are instructed to collect two level spoons of stool which are added to the Alcorfix®-containing portion of the tube. Alcorfix® is new to North American markets, and is an alcohol-based fixative (ethanol, PVA, isopropanol, methyl alcohol, with acetic acid, glycerin, and zinc sulfate) that is compatible with both wet mount preparations and permanent stains; thus absolving the need for two separate collection media. This tube and fixative combination are intended to centralize the specimen collection, fixation, and concentration in a single device while providing a streamlined workflow for the laboratory. One perceived limitation with the Parasep® tubes is the fact that the tubes are designed to concentrate the entire content of the specimen, which may pose a problem for laboratories that perform quantification of *Blastocystis hominis* and permanent smears from unconcentrated stool only.

Though the Parasep® tubes have been commercially available with other fixatives previously in various world markets, this study provides the first in-depth evaluation of Parasep® tubes with AlcorFix® fixative in a large parasitology laboratory. The three primary goals of this study were to: investigate the effectiveness of Alcorfix® versus conventional PVA and formalin fixation; compare the concentration

Journal of Clinical Microbiology

efficiency of Parasep tubes to the SpinCon® concentration method; and to determine if quantification of parasites (e.g. *Blastocystis hominis*) can be accurately performed from Parasep® concentrated stools compared to conventional quantification from non-concentrated specimens.

METHODS

Specimen concentration optimization

The optimal concentration speed was determined by comparing two centrifugation speeds (200 *x g* and 400 *x g*) using the assembled Parasep® tube in a Sorval ST40 centrifuge equipped with a TX-1000, 209mm rotor. Unconcentrated specimens previously collected in 10% formalin and polyvinyl alcohol (PVA) containing zinc, copper, or mercury (Meridian Biosciences, Cincinnati, OH) and identified as containing protozoal cysts or trophozoites were processed in the Parasep® tube. A 3ml sample was aliquotted from the unconcentrated formalin and PVA samples prior to centrifugation, and two subsequent 3ml aliquots were removed from each fixative and concentrated in the Parasep® tubes at two centrifugal speeds listed above. Parasite morphology (specificity) and relative abundance (sensitivity, defined as 1+ through 4+) were scored blindly by two independent parasitologists for the unconcentrated stool, and concentrated stool processed at each speed. The assessments were defined as: 1+ = $\leq$ 3organisms/high power field 1000X (HPF), 2+ 4-6 organisms/HPF, 3+ 7-9 organism/HPF, and 4+ $\geq$ 10 organisms/HPF.

Concentration efficiency

Forty-seven specimens that were previously identified by conventional O&P as containing parasites were processed as per standard laboratory protocol using a SpinCon® (Meridian Biosciences, Cincinnati, OH) concentration device for both the PVA and formalin specimens. Three specimens that were negative were also included in the study set. SpinCon® concentrators do not utilize solvents for

concentration such as ether or ethyl acetate, but Triton-X100 is added by the laboratory to the specimen as a surfactant before concentration. SpinCon® filters utilize passive and centrifugal filtration through a series of two filters housed within an assembled filter tube device. The filtration requires manual transfer of 3ml of stool from the collection device to the filtration assembly, followed by physical mixing of particulates and addition of 2ml of saline to facilitate initiation of the concentration process. Specimens are then centrifuged at 500xg for 10 minutes, and the supernatant is discarded, yielding a small pellet of concentrate. Parasep® tubes concentrate a total of 3ml of fixative with stool, and the Alcorfix® contains the appropriate concentration of Triton-X100 at the time of stool collection. The tubes are vortexed briefly to mix the contents, then centrifuged at 400 *x g* for 2 minutes. The supernatant is discarded, leaving a pellet of concentrate for analysis that is amenable to multiple preparations for repeat testing or teaching purposes, similar to SpinCon®.

An equal volume of fixed stool (3mL) was added to the SpinCon® filter and the Parasep® tubes for equal specimen volume comparison. The sediments from both concentration methods were then analyzed by trichrome stain (permanent slide) and wet mount preparation with Lugul's iodine for contrast (3). The two parasitologists were blind to the original results and the concentration method.

Clinical performance studies for Mini Parasep® SF tubes

Parasep® tubes containing Alcorfix® were distributed to patients at the University of Utah hospitals and clinics between February and September of 2014 in accordance with our institution's IRB board. Patients were instructed to collect stool in the standard PVA and formalin tubes in addition to the Parasep® tube for each unique stool specimen submitted for routine O&P testing. Samples were processed by the parasitology laboratory concurrently using SpinCon® concentration as well as the optimized Parasep® concentration. The O&P results for the different collection devices and fixatives were compared in real-time for clinical performance (ability to identify parasites by staining and

morphology). Specimens were read by the clinical parasitology laboratory in real-time, and were blinded to the concentration method and fixative used. Overfilled vials were not included in the evaluation, as the fixation could be compromised. Incompletely filled vials were excluded to remove any potential inequalities in sensitivity between the two collection methods.

Modified acid-fast stain compatibility

A total of $1x10^9$ *Cryptosporidium parvum* oocysts passaged through bovines were purchased commercially from Waterborne Inc. (New Orleans, LA), and spiked into stool submitted for routine fecal chemistries. Three milliliters of stool was added to Parasep® tubes containing Alcorfix® or 10% formalin, and processed according to the optimized concentration. The concentrate was prepared for Modified Acid fast staining (3).

Modified trichrome stain compatibility

A total of $1x10^8$ *Encephalitozoon cuniculi* and *Encephalitozoon intestinalis* spores in PBS were purchased separately from Waterborne Inc. (New Orleans, LA), and spiked into stool submitted for routine fecal chemistries. Stool was added to Alcorfix® or 10% formalin, and an aliquot was sampled for modified trichrome (chromotrope 2R) using the Ryan Blue staining method and processed according to the optimized concentration (3).

Workflow efficiency comparison

Stool was added to 10 Parasep® tubes and 10 PVA and formalin tube sets and a second workflow was performed for a batch of 30 tubes/tube-sets to mimic different laboratory test volumes. Three independent technicians from the parasitology processing laboratory performed concentration for each batch (n= 10 and 30). Each step was timed and the total time to prepare a run with each

fixative/concentration method was recorded. The mean processing time for each batch and standard deviation was calculated.

RESULTS

Concentration optimization

The optimal centrifugation speed for the Parasep® tube was determined by comparing two different concentration speeds, 200 *x g* and 400 *x g*. The specimens were scored according to relative abundance of protozoa in the specimen (Table 1). The specimen was also evaluated without concentration in an attempt to determine whether protozoa that are perceived to be more fragile and subject to lysis or deformation (*e.g. Blastocystis hominis*) were detectable in similar abundance before and after centrifugation at either speed, thus providing a more streamlined workflow to remove the necessity of sampling stool before concentration. Ten specimens preserved in formalin and PVA were tested, including 7 specimens containing varying quantities of *B. hominis* (Table 1.). Overall the two centrifugation speeds were comparable in yield of protozoa on either the trichrome stain or wet mount examination. The pellet yielded from 400 *x g* centrifugation yielded less loss of material from pour-off than was observed with 200 *x g.* Correspondingly, there was no noticeable loss in sensitivity in the form of protozoal recovery. The increased yield also allows for more preparations to be made from a single concentrate, and therefore this centrifugation speed was applied to the remainder of the study. Sampling stool without concentration did not reveal any consistent increase in yield for *B. hominis* (Table 1).

Concentration efficiency

With an optimized concentration protocol established, a direct comparison of the Parasep® tube with the standard concentration technique performed in the laboratory (SpinCon®) was performed on

50 stool specimens that were previously collected in formalin and PVA, representing approximately two months of testing. Forty-seven of these specimens were known to be positive for various protozoa (three negative specimens were also included) spanning multiple genera and were blindly analyzed by two parasitologists (Table 2). Forty-nine of fifty specimens had matching results identified in the final report based on examination of the trichrome stain and wet mount preparation for each concentration method. The discrepant specimen was positive for *B. hominis* and *Entamoeba coli* by Parasep® concentration but only positive for *B. hominis* by SpinCon® (Table 2, sample F18). The *Entamoeba coli* were verified by both parasitologists for resolution. A single specimen that contained an interfering substance rendered the wet mount preparation unreadable with iodine contrast for both concentration methods tested.

Clinical evaluation

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 were provided for O&P collection which included the standard PVA and formalin as well as a Parasep® tube. Submission of the Parasep® tube was optional for the patient, and a majority of tubes were either returned to the laboratory unfilled or had been discarded by the patient. A total of 26 specimens were submitted for testing in which both fixatives were filled with stool at the required specimen volumes indicated by the respective manufacturers. Three specimens contained parasites of the 26 submissions meeting inclusion criteria (Figure 2). The specimen from patient 1 contained 1+ *B. hominis*, *E. coli,* and *E. nana*, (Figure 2A-F) each of which was identified in both fixative concentrates by two independent technologists. The morphology was considered acceptable by both technologists for both fixative/concentration methods. Stool from patient 2 contained 1+ *B. hominis* which was identified by both technologists in formalin, but only one technologist in the Alcorfix® specimen. Upon re-

Journal of Clinical Microbiology

examination of the samples, *B. hominis* was seen with variable morphology in both the formalin and Alcorfix wet-mount but the organisms were rare in both preparations (Figure 2G-H). Stool from the third patient contained *Giardia lamblia* cysts and trophozoites in both preparations with readily identifiable morphology, however the stain retention and morphology was considered overall more consistent in Alcorfix® by both technologists (Figure 2 I-J).

Analytical evaluation of modified acid-fast staining

Alcorfix® containing Parasep® tubes showed acceptable performance for conventional trichrome stain and wet mount evaluation; however no coccidian parasites were encountered in our prospective co-collection study. To test whether Alcorfix® would be compatible with modified acid fast stain and also fix the oocysts such that morphology of the coccidian is retained, live *Cryptosporidium parvum* oocysts were procured and spiked into fresh stool. The stool was separated into vials containing formalin and Alcorfix® and prepared for microscopic examination with a permanent acid-fast stain after concentration in Parasep® tubes. The morphology of the oocysts was maintained in both fixatives, with slightly rounder morphology retained in formalin, however many of the oocysts in each fixative failed to retain the modified acid-fast stain and were only visible as "ghosts". Alcorfix showed comparatively better stain retention than formalin in these simulated specimens (Figure 3A and B), however both fixatives retained the stain with recognizable morphology nonetheless.

Analytical evaluation of modified trichrome staining

In a final effort to ensure Alcorfix® with Parasep® tubes can serve universal stool parasite interrogation, live spores of microsporidia 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).

Workflow study

One perceived advantage of Parasep® tubes is the potential to improve the current workflow for processing O&P specimens by converging from two collection vials to one tube. Another major advantage of these tubes is the built-in filtration device that is housed within the assembled tube which allows direct centrifugation and reduced hands-on time (eliminate transfer of stool to a separate concentration device). Three laboratory technicians with extensive experience in processing O&P specimens independently processed batches of 10 or 30 O&Ps prepared in PVA/formalin tubes or Parasep® tubes. Each step was timed and recorded, and the average and standard deviation was calculated for each batch size and concentration method (Table 3). The average time to prepare a run of 30 O&P specimens was 51 minutes and 46 seconds (4:25 standard deviation) using SpinCon® concentration for PVA/formalin, versus 19:45 (standard deviation 1:52) for Parasep®. This difference in workflow represents an average time savings of 32:01. For a run of 10 O&P specimens, the average time for SpinCon® concentration was 27:41 (standard deviation 1:58), versus 10:02 (standard deviation 0:44) for Parasep® concentration. This represents an average time savings of 17:39.

DISCUSSION

In the last 20 years, considerable efforts have been made by commercial manufacturers of stool fixatives for parasite interrogation to remove formalin and mercuric PVA fixatives from the laboratory and patient collection site. These advancements have allowed safer specimen collection and processing for both patients and laboratory staff. An additional advantage to these fixatives is reduced disposal fees for the laboratory (related specifically to formalin waste disposal) and elimination of formalin exposure monitoring for staff in the parasitology laboratory. In the same period that these advancements in fixation and specimen collection have arisen, another trend has developed in clinical microbiology laboratories in several countries; consolidation of testing in centralized laboratories or

outsourcing of labor intensive/low revenue tests to larger reference laboratories. O&P examinations represent a very common test that has become centralized in distal sites from the primary collection. In our large national reference laboratory, O&P examinations have grown to more than 60,000 examinations per year, which represents approximately 41% increase over 10 years. This is a trend similarly experienced in other large centralized laboratories in the USA and Canada (personal communications). This increased demand on laboratories for O&P testing also poses significant challenges in meeting clinically reasonable turn-around times as well as placing increased demand on the most experienced parasitologists in the laboratory; who are becoming less numerous in the current workforce. Any advancement in O&P examinations likely will produce immediate benefits to laboratories that perform high volumes of O&P examinations. The most targetable area for improvement is in processing of specimens and preparation of concentrates for microscopy.

Parasep® tubes provide an attractive option to parasitology laboratories to improve processing of specimens, however the tubes and Alcorfix® fixative are relatively new to the North American market; as such, no in depth evaluation has been performed to establish a universal comparison to methods commonly employed in this broad geographic region. One European study did previously compare ethyl-acetate precipitation to Parasep® solvent-free tubes, and found better ova recovery using the standard ethyl-acetate method, however this does not directly compare to the methodologies utilized in most US laboratories (8). This study aimed to evaluate the single vial, all-in-one design of the Parasep® tube for implementation in parasitology laboratories in the United States compared to current standard methodologies. In order to consider implementation of these tubes and fixative, the performance characteristics needed to match or exceed the current “gold standard”. Though many laboratories in North America may use different permutations of O&P testing, the core processes are mostly superimposable or based on conserved methods (3).

Journal of Clinical Microbiology

The first phase of this study aimed to maximize the performance of Parasep® tubes using different concentration speeds in previously identified and fixed stool specimens in PVA/formalin. Parasep tubes have been used in Europe for many years with a recommended centrifugation speed of 200 *x g*, while the recommended centrifugation for use in the United States for trichrome stain compatibility was 500 *x g*. Using cultured *Giardia*, we found that slight distortion was seen at 500 *x g* (Data not shown). Therefore we aimed to test the European centrifugation conditions compared to 400 *x g* in an effort to determine what centrifugation would maximize the sensitivity and specificity (morphology) of the parasites. The 400 *x g* concentration speed produced comparable yield of parasites to 200 *x g* with no loss in parasite morphology. The concentrated pellet produced from a 400 *x g* concentration was also advantageous, as Parasep® tubes concentrate the entire collected specimen at once, leaving no residual stool for subsequent concentrations. The increased pellet yield negates this limitation by providing additional biomass in order to make multiple trichrome stains and wet mount preparations as may be needed without affecting sensitivity. This increased pellet yield is also valuable for larger parasitology laboratories that utilize residual specimens for teaching.

Of significant importance was to determine if the faster concentration speed would allow for accurate enumeration of *B. hominis*, the most common gastrointestinal parasite encountered in North America (9). Many laboratories currently sample the unconcentrated stool in order to detect and semi-quantify *B. hominis* due to concerns that concentration will change the relative abundance and morphology of the parasites (or lyse the fragile trophozoites)(3). Seven specimens containing *B. hominis* were evaluated in order to determine the necessity to sample from unconcentrated stool in advance of concentration, and no consistent differences were seen between sampling before or after concentration at either concentration speed. No major discrepancy in semiquantitation was seen (*i.e* 1+ versus 4+); rather the differences in semiquantitative measure were arguably of dubious clinical significance (*i.e* 1+ versus 2+). This suggests that sampling from the unconcentrated stool for trichrome

stain may not be a necessary procedure for adequate trophozoite recovery and enumeration. This finding also further streamlines the processing of Parasep® tubes, preventing an additional sampling step in advance of concentration and questions long-standing recommendations that may not be absolutely necessary in practice.

A direct comparison of Parasep® concentration versus the laboratory's current process of concentration using SpinCon® filters was conducted using PVA/formalin specimens that were previously identified as containing protozoa. Overall the two methods were comparable, with only one specimen showing unequal test interpretations. In this specimen, protozoa were identified in the Parasep® preparation that were absent in the SpinCon® preparation despite multiple reviews of the preparations. It should be noted that these differences could be due to low abundance of parasites and sampling error of non-homogenous matrices. Overall the majority of specimens had seemingly identical evaluations, which supports the equivalence of the filtration methods. Of interest, the specimen that contained an interfering substance was equally problematic for both concentration methods, and may not be avoided in certain specimens which are occasionally encountered during clinical testing.

Collection of patient samples in Parasep® tubes containing Alcorfix® in tandem with PVA/formalin represented a significant challenge to the study. ARUP laboratories serves hospitals in 49 of 50 states in the United States, each of which can submit O&P samples to our laboratory in fixatives of their choice, though the fixatives supplied by ARUP are PVA with copper and 10% formalin, which represents the majority of submissions the laboratory receives. At the time of this study, no hospitals that our laboratory serves were using Parasep® tubes. In order to effectively attain co-collection in our standard collection tubes as well as the Parasep®, we issued both collection tubes in a standard stool collection kit to the University of Utah hospitals and clinics, for which ARUP serves as the primary/on-site microbiology laboratory. The University of Utah hospital and clinics serves primarily the greater Salt

Lake valley of northern Utah, for which the geography is high desert, chaparral, and mountainous terrain with little ground water and very low humidity (~10-20% relative humidity). Expectedly, the rate of local parasite acquisition is very low based on historic O&P and stool antigen positivity rates in the laboratory ((7) and unpublished data). Despite these inherent limitations and poor patient compliance with full volume stool collection, three of the twenty-six specimens submitted over the course of the study were positive for protozoa, representing four different genera of protozoal parasites each with distinct morphological traits that are necessary for accurate identification (*Endolimax nana*, *Entamoeba coli*, *Blastocystis hominis*, and *Giardia lamblia*). Each of the organisms was identified in each fixative by the testing technologists, largely with very comparable morphology and stain quality. The specimen containing *Giardia* did reveal better stain retention and morphology using Alcorfix® with Parasep® tube concentration, however this single observation is not sufficient to claim superiority. The laboratory however did not have any difficulty reading the positive specimens for either preparation method. Also of note, the parasitology technologists did report that the overall slide clarity was superior with concentration and fixation with Parasep®/Alcorfix®, primarily attributed to a cleaner background and less large particulate fecal matter in the preparations. This could possibly account for the improved stain retention that was noted for the Alcorfix® specimens since less stain was absorbed by the background debris; an observation our laboratory has documented particularly when smears are made too thick (unpublished data).

In the course of our study, none of the 26 specimens had modified acid fast or modified trichrome staining ordered. As a result our sample set did not have coccidian parasites or microsporidia represented (no specimens were suspicious by trichrome stain for coccidia or microsporidia as well). For a laboratory to streamline to a single vial and eliminate formalin from the laboratory, the fixative must allow these groups of parasites to be detected when the specific staining is indicated. The simulated specimens for *Cryptosporidium, E. cuniculi,* and *E. intestinalis* contained live organisms, which allowed us

to simulate collection from a patient, timely fixation in the collection tubes, and staining with the indicated stains. Of note for *Cryptosporidium*, many ghost cells were seen. Ghost cells are more commonly seen with *Cyclospora* on modified acid fast stains; however ghost cells of *Cryptosporidium* have regularly been encountered in specimens in our laboratory (unpublished data). The preparation for both formalin and Alcorfix® showed ghost cells, however the morphology of the oocysts was conserved, and the oocysts that did stain showed predictable stain retention. Given that more oocysts stained on the Alcorfix® preparation than the formalin, one can conclude that the compatibility is at least equivalent to the gold standard. Microsporida showed predictable staining and morphology, with slightly better stain retention for the Alcorfix fixed specimen, further suggesting that substituting formalin with Alcorfix® is a viable alternative providing true single vial testing for microscopic ova and parasite examinations.

A significant advantage of Parasep® tubes is the increased productivity that can be achieved by the shorter time for specimen preparation (9). The workflow study performed in this investigation showed time savings of over half an hour for thirty specimens, and seventeen minutes for ten specimens. The majority of the time savings are gained in not having to transfer specimens to subsequent filters, not having to print and label multiple independent tubes/filters in order to maintain secure patient identification, and reducing the centrifugation time by 80% (or 8 minutes). For laboratories processing moderate-to-high volumes of O&P tests, this represents a significant advantage over the current laborious concentration procedures used in many laboratories. In fact, for a batch of 30 specimens, the hands-on time savings were over 30 minutes. Given the centralization of O&P examinations in many reference and regional laboratories, the Parasep® tubes could provide significant benefit to laboratories that have experienced increasing volumes of these tests. A future area of exploration underway in our laboratory is automating the tube disassembly during processing, in an

attempt to reduce repetitive stress injuries attributable to unscrewing caps. This would further reduce the processing time.

Our study has several acknowledged limitations of note. First, our co-collection study yielded only 3 specimens containing a total of 5 parasites of a total of 26 appropriately enrolled specimen sets, though this represents a higher positivity rate for O&Ps from northern Utah than is normally encountered (historically <1% positivity, unpublished data). This increased positivity could be a result of receiving specimens primarily from two outpatient clinics that routinely serve return travelers, religious missionaries, and immigrants seeking care for non-emergency illnesses. A second limitation is that our specimen collection study did not encounter coccidian parasites or microsporidia. We addressed this limitation through incorporation of live parasite suspensions to bolster the analytical evaluation of these parasites and test the fixation ability of Alcorfix®. Third, we did not test any ova in our study due to having not received any ova in the course of our co-collection study or in the retrospective PVA/formalin specimens. One specimen did contain *Strongyloides* rhabiditoid larvae (Table 2), and it was equally recovered using both concentration methods. Previous studies have shown recovery of ova using Parasep® tubes is achievable (2, 9).

This study provides a comprehensive evaluation of the Mini Parasep® SF tube with Alcorfix® fixative in a large national reference laboratory. This combination of tube and fixative represents a viable single-vial option for laboratories in pursuit of a formalin-free, streamlined collection and processing capabilities. The fixative is compatible with all iterations of microscopic parasite examination and staining from one concentrated sediment, alleviating the necessity of sampling stool from unconcentrated samples. Future studies will evaluate the compatibility with antigen detection and molecular methods. Finally, this tube/fixative combination allows for comprehensive parasite detection

with improved workflow advantages for processing of stool parasite examinations, particularly in laboratories with increasing test volumes.

379 Acknowledgements:

380 We are grateful to the staff members in Parasitology and Infectious Disease Rapid Testing laboratories at
381 ARUP laboratories for their hard work and dedication to this project. We also would like to thank the
382 University of Utah Hospital and Clinics for assisting us in the co-collection study.

383 We thank Apacor for providing reagents for collection and testing. MRC and BAC received honoraria
384 from Apacor.

385 These data were presented, in part, at the 2015 ECCMID Meeting in Copenhagen, Denmark and the
386 2015 American Society for Microbiology General Meeting in New Orleans, Louisiana.

387

388

Journal of Clinical Microbiology

389 REFERENCES

390 1. **Fedorko, D. P., E. C. Williams, N. A. Nelson, T. D. Mazyck, K. L. Hanson, and C. P. Cartwright.**
391 2000. Performance of para-Pak Ultra ECOFIX compared with Para-Pak Ultra formalin/mercuric
392 chloride-based polyvinyl alcohol for concentration and permanent stained smears of stool
393 parasites. Diagn Microbiol Infect Dis **37:**37-9.
394 2. **Funk, A. L., S. Boisson, T. Clasen, and J. H. Ensink.** 2013. Comparison of Kato-Katz, ethyl-acetate
395 sedimentation, and Midi Parasep(R) in the diagnosis of hookworm, Ascaris and Trichuris
396 infections in the context of an evaluation of rural sanitation in India. Acta Trop **126:**265-8.
397 3. **Garcia, L. S., and H. D. Isenberg.** 2010. Clinical microbiology procedures handbook, 3rd ed. ASM
398 Press, Washington, DC.
399 4. **Garcia, L. S., and R. Y. Shimizu.** 1998. Evaluation of intestinal protozoan morphology in human
400 fecal specimens preserved in EcoFix: comparison of Wheatley's trichrome stain and EcoStain. J
401 Clin Microbiol **36:**1974-6.
402 5. **McHardy, I. H., M. Wu, R. Shimizu-Cohen, M. R. Couturier, and R. M. Humphries.** 2014.
403 Detection of intestinal protozoa in the clinical laboratory. J Clin Microbiol **52:**712-20.
404 6. **Pietrzak-Johnston, S. M., H. Bishop, S. Wahlquist, H. Moura, N. D. Da Silva, S. P. Da Silva, and**
405 **P. Nguyen-Dinh.** 2000. Evaluation of commercially available preservatives for laboratory
406 detection of helminths and protozoa in human fecal specimens. J Clin Microbiol **38:**1959-64.
407 7. **Polage, C. R., G. J. Stoddard, R. T. Rolfs, and C. A. Petti.** 2011. Physician use of parasite tests in
408 the United States from 1997 to 2006 and in a Utah Cryptosporidium outbreak in 2007. J Clin
409 Microbiol **49:**591-6.
410 8. **Saez, A. C., M. M. Manser, N. Andrews, and P. L. Chiodini.** 2011. Comparison between the Midi
411 Parasep and Midi Parasep Solvent Free (SF) faecal parasite concentrators. J Clin Pathol **64:**901-4.
412 9. **Zeeshan, M., A. Zafar, Z. Saeed, S. Irfan, Z. A. Sobani, S. Shakoor, and M. A. Beg.** 2011. Use of
413 "Parasep filter fecal concentrator tubes" for the detection of intestinal parasites in stool samples
414 under routine conditions. Indian J Pathol Microbiol **54:**121-3.

415

416

417 Table 1. Concentration optimization for PVA/formalin samples containing known protozoal parasites

418 using Parasep® concentrator tubes.

Sample	Centrifugation (*x g*)	Wet mount result	Trichrome stain result
1	Unconcentrated	2+ *B. hominis*	Uninterpretable
	200	1+ *B. hominis*	Uninterpretable
	400	1+ *B. hominis*	Uninterpretable
2	Unconcentrated	2+ *Chilomastix*	1+ *Chilomastix*
	200	1+ *Chilomastix*	2+ *Chilomastix*
	400	1+ *Chilomastix*	3+ *Chilomastix*
3	Unconcentrated	1+ *B. hominis* rare trophozoites	1+ *B. hominis*
	200	1+ *B. hominis* 1+ *E. nana*	1+ *B. hominis*
	400	1+ *B. hominis* 1+ *E. nana*	1+ *B. hominis*
4	Unconcentrated	1+ *B. hominis*	Negative
	200	1+ *B. hominis*	Negative
	400	1+ *B. hominis*	Negative
5	Unconcentrated	1+ *B. hominis*	1+ *B. hominis*
	200	1+ *B. hominis*	1+ *B. hominis*
	400	1+ *B. hominis*	1+ *B. hominis*
6	Unconcentrated	1+ trophozoites	Rare *E. histolytica/dispar*
	200	1+ trophozoites	1+ *E. histolytica/dispar*
	400	1+ trophozoites	1+ *E. histolytica/dispar*
7	Unconcentrated	1+ *B. hominis*	Rare *B. hominis*
	200	1+ *B. hominis*	Rare *B. hominis*
	400	1+ *B. hominis*	1+ *B. hominis*
8*	Unconcentrated	2+ *B. hominis* 1+ trophozoites	1+ *B. hominis* Rare *E. coli*
	200	3+ *B. hominis* 1+ trophozoites	1+ *B. hominis* 1+ *E. coli*
	400	2+ *B. hominis* 1+ trophozoites	1+ *B. hominis* 1+ *E. coli*
9**	Unconcentrated	4+ *B. hominis* 1+ trophozoites	1+ *B. hominis* 1+ *E. coli*
	200	3+ *B. hominis* 1+ trophozoites	1+ *B. hominis* 1+ *E. coli*
	400	2+ *B. hominis* 1+ trophozoites	1+ *B. hominis* 1+ *E. coli*
10	Unconcentrated	1+ *Giardia* cysts	Rare *Giardia*

200	1+ *Giardia* cyst and trophozoites	1+ *Giardia*
400	1+ *Giardia* cysts	1+ *Giardia*

419

420 Table 2. Comparison of concentration efficiency of SpinCon® and Parasep® concentration techniques.
421 Specimens were fixed previously in PVA and formalin and identified according to the standard
422 laboratory operating procedures.

Study ID	SpinCon® Final identification	Parasep® Final Identification
F1	1+ *B. hominis*	1+ *B. hominis*
F2	*E. coli*	*E. coli*
F3	Negative	Negative
F4	1+ *B. hominis*	1+ *B. hominis*
F5	1+ *B. hominis* Dientamoeba fragilis	1+ *B. hominis* Dientamoeba fragilis
F6	Negative	Negative
F7	Negative	Negative
F8	*D. fragilis*	*D. fragilis*
F9	*E. coli*	*E. coli*
F10	2+ *B. hominis*	2+ *B. hominis*
F11	*E. nana* 2+ WBC	*E. nana* 2+ WBC
F12	3+ B. *hominis*	3+ B. *hominis*
F13	2+ *B. hominis*	2+ *B. hominis*
F14	2+ *B. hominis* *E. nana*	2+ *B. hominis* *E. nana*
F15	*Strongyloides* rhabiditoid larvae	*Strongyloides* rhabiditoid larvae
F16	*D. fragilis*	*D. fragilis*
F17	*E. nana*	*E. nana*
F18	2+ *B. hominis*	2+ *B. hominis* ***E. coli***
F21	*Giardia*	*Giardia*
F22	1+ *B. hominis* *E. nana*	1+ *B. hominis* *E. nana*
F23	*D. fragilis*	*D. fragilis*
F24	*Giardia* 3+ WBC	*Giardia* 4+WBC
F25	1+ *B. hominis*	1+ *B. hominis*
F26	1+ *B. hominis* *E. nana*	1+ *B. hominis* *E. nana*
F27	1+ *B. hominis* *E. nana*	1+ *B. hominis* *E. nana*
F28	1+ *B. hominis* *E. nana*	1+ *B. hominis* *E. nana*
F29	1+ *B. hominis* *E. nana*	1+ *B. hominis* *E. nana*

F30	*E. coli* *E. nana* 1+ WBC	*E. coli* *E. nana* 1+ WBC
F31	*E. nana*	*E. nana*
F32	*E. nana*	*E. nana*
F33	*E. nana*	*E. nana*
F34	1+ *B. hominis* *D. fragilis* 1+ WBC	1+ *B. hominis* *D. fragilis*
F35	*D. fragilis*	*D. fragilis*
F36	*E. nana*	*E. nana*
F37	2+ *B. hominis*	2+ *B. hominis*
F38	1+ *B. hominis* *Giardia* *E. histolytica/dispar*	1+ *B. hominis* *Giardia* *E. histolytica/dispar*
F39	*Giardia*	*Giardia*
F40	1+ *B. hominis*	1+ *B. hominis*
F41	*D. fragilis*	*D. fragilis*
F42	1+ *B. hominis*	1+ *B. hominis*
F44	1+ *B. hominis*	1+ *B. hominis*
F45	1+ *B. hominis* *E. nana* *D. fragilis*	1+ *B. hominis* *E. nana* *D. fragilis*
F46	1+ *B. hominis*	1+ *B. hominis*
F47	*E. coli* *E. nana*	*E. coli* *E. nana*
F48	4+ *B. hominis*	4+ *B. hominis*
F49	4+ *B. hominis*	4+ *B. hominis*
F50	3+ *B. hominis* *E. nana* *Entamoeba hartmanni* *E. histolytica/dispar* *Giardia*	3+ *B. hominis* *E. nana* *Entamoeba hartmanni* *E. histolytica/dispar* *Giardia*
F51	*E. histolytica/dispar* *E. hartmanni*	*E. histolytica/dispar* *E. hartmanni*
F52	*B. hominis*	*B. hominis*
F53	*D. fragilis* *2+ B. hominis*	*D. fragilis* *2+ B. hominis*

423 WBC = white blood cell

424 Table 3. Workflow comparison for SpinCon® concentration versus Mini Parasep® SF concentration.

	30 run-Formalin/PVA		10 run-Formalin/PVA	
	Processing time		Processing time	
PVA/Formalin Processing steps	**Mean**	**Std Dev**	**Mean**	**Std Dev**
Name check - 2 tubes/patient	0:02:51	0:00:33	0:01:32	0:00:16
Label making	0:04:40	0:00:08	0:02:25	0:00:24
Label each funnel & tube (2 funnels/patient)	0:11:59	0:00:17	0:04:20	0:00:37
Pour 3 ml of stool into funnel	0:30:44	0:03:46	0:11:04	0:01:52
Manually break up formed stool				
Label funnel (PVA or Formalin)				
10 min spin at 500 x g	0:43:19	0:03:55	0:22:58	0:02:04
Pour-off supernatant	0:49:13	0:04:28	0:25:32	0:02:00
Build Run - Final time	**0:51:46**	0:04:25	**0:27:41**	0:01:58

	30 run-Parasep		10 run-Parasep	
	Processing time		Processing time	
Parasep® Processing steps	**Mean**	**Std Dev**	**Mean**	**Std Dev**
Name check - 1 vial	0:01:54	0:00:28	0:00:50	0:00:07
Label making	0:02:44	0:00:36	0:01:08	0:00:06
Label Parasep® tube	0:06:08	0:00:52	0:02:08	0:00:17
Briefly vortex and invert	0:08:11	0:00:19	0:02:44	0:00:27
2 min spin at 400 x g	0:12:08	0:00:32	0:06:07	0:00:46
Pour-off supernatant	0:17:37	0:01:41	0:08:02	0:00:44
Build run - Final time	**0:19:45**	0:01:52	**0:10:02**	0:00:44

425

FIGURE TITLES:

Figure 1. Mini Parasep® SF collection tube showing unassembled (left) tube set submitted to patients and assembled tube received from patient (right). Arrow indicates conical sediment collection tube, arrowhead indicates flat bottom collection tube (shown without fixative), * indicates screw cap, # indicates vertical filtration spoon.

Figure 2. Positive stool specimens co-collected in PVA/formalin and Alcorfix®. Representative protozoa are shown for both fixative types. Trichrome stained images (patient 1 & 3) were captured at 1000X magnification with an oil immersion lens and wet-mount images (patient 2) were captured at 400X magnification.

Figure 3. Representative images of stool specimens spiked with live coccidia (*Cryptosporidium parvum,* A-B) or microsporidia (*Encephalitozoon intestinalis* [C-D], *Encephalitozoon cuniculi* [E-F]), fixed in formalin or Alcorfix®, and stained with modified acid-fast stain (A-B) or modified trichrome stain (C-F). All images were captured at 1000X magnification.