

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

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10 Running head: Improved ova and parasite specimen processing

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14 **Abstract:**

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

## 34 INTRODUCTION

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

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

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

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

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

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

### 83 METHODS

#### 84 Specimen concentration optimization

85 The optimal concentration speed was determined by comparing two centrifugation speeds (200  
86  $\times g$  and 400  $\times g$ ) using the assembled Parasep® tube in a Sorval ST40 centrifuge equipped with a TX-  
87 1000, 209mm rotor. Unconcentrated specimens previously collected in 10% formalin and polyvinyl  
88 alcohol (PVA) containing zinc, copper, or mercury (Meridian Biosciences, Cincinnati, OH) and identified  
89 as containing protozoal cysts or trophozoites were processed in the Parasep® tube. A 3ml sample was  
90 aliquotted from the unconcentrated formalin and PVA samples prior to centrifugation, and two  
91 subsequent 3ml aliquots were removed from each fixative and concentrated in the Parasep® tubes at  
92 two centrifugal speeds listed above. Parasite morphology (specificity) and relative abundance  
93 (sensitivity, defined as 1+ through 4+) were scored blindly by two independent parasitologists for the  
94 unconcentrated stool, and concentrated stool processed at each speed. The assessments were defined  
95 as: 1+ =  $\leq 3$  organisms/high power field 1000X (HPF), 2+ 4-6 organisms/HPF, 3+ 7-9 organism/HPF, and  
96 4+  $\geq 10$  organisms/HPF.

#### 97 Concentration efficiency

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

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

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

#### 117 **Clinical performance studies for Mini Parasep® SF tubes**

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

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

#### 129 **Modified acid-fast stain compatibility**

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

#### 135 **Modified trichrome stain compatibility**

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

#### 141 **Workflow efficiency comparison**

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

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

148 RESULTS

#### 149 **Concentration optimization**

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

#### 165 **Concentration efficiency**

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



168 50 stool specimens that were previously collected in formalin and PVA, representing approximately two  
169 months of testing. Forty-seven of these specimens were known to be positive for various protozoa  
170 (three negative specimens were also included) spanning multiple genera and were blindly analyzed by  
171 two parasitologists (Table 2). Forty-nine of fifty specimens had matching results identified in the final  
172 report based on examination of the trichrome stain and wet mount preparation for each concentration  
173 method. The discrepant specimen was positive for *B. hominis* and *Entamoeba coli* by Parasep®  
174 concentration but only positive for *B. hominis* by SpinCon® (Table 2, sample F18). The *Entamoeba coli*  
175 were verified by both parasitologists for resolution. A single specimen that contained an interfering  
176 substance rendered the wet mount preparation unreadable with iodine contrast for both concentration  
177 methods tested.

#### 178 **Clinical evaluation**

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

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

#### 196 **Analytical evaluation of modified acid-fast staining**

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

#### 208 **Analytical evaluation of modified trichrome staining**

209 In a final effort to ensure Alcorfix® with Parasep® tubes can serve universal stool parasite  
210 interrogation, live spores of microsporidia were procured, spiked into stool, and fixed in both 10%  
211 formalin and Alcorfix® separately. Modified trichrome staining was performed on both fixatives and  
212 evaluated microscopically. Both preparations showed conserved morphology predictable for  
213 microsporidia, as well as adequate stain retention for both *E. intestinalis* and *E. cuniculi* (Figure 3C-F).

214 **Workflow study**

215 One perceived advantage of Parasep® tubes is the potential to improve the current workflow for  
216 processing O&P specimens by converging from two collection vials to one tube. Another major  
217 advantage of these tubes is the built-in filtration device that is housed within the assembled tube which  
218 allows direct centrifugation and reduced hands-on time (eliminate transfer of stool to a separate  
219 concentration device). Three laboratory technicians with extensive experience in processing O&P  
220 specimens independently processed batches of 10 or 30 O&Ps prepared in PVA/formalin tubes or  
221 Parasep® tubes. Each step was timed and recorded, and the average and standard deviation was  
222 calculated for each batch size and concentration method (Table 3). The average time to prepare a run of  
223 30 O&P specimens was 51 minutes and 46 seconds (4:25 standard deviation) using SpinCon®  
224 concentration for PVA/formalin, versus 19:45 (standard deviation 1:52) for Parasep®. This difference in  
225 workflow represents an average time savings of 32:01. For a run of 10 O&P specimens, the average  
226 time for SpinCon® concentration was 27:41 (standard deviation 1:58), versus 10:02 (standard deviation  
227 0:44) for Parasep® concentration. This represents an average time savings of 17:39.

228 **DISCUSSION**

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

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

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

260 The first phase of this study aimed to maximize the performance of Parasep® tubes using  
261 different concentration speeds in previously identified and fixed stool specimens in PVA/formalin.  
262 Parasep tubes have been used in Europe for many years with a recommended centrifugation speed of  
263 200 x g, while the recommended centrifugation for use in the United States for trichrome stain  
264 compatibility was 500 x g. Using cultured *Giardia*, we found that slight distortion was seen at 500 x g  
265 (Data not shown). Therefore we aimed to test the European centrifugation conditions compared to 400  
266 x g in an effort to determine what centrifugation would maximize the sensitivity and specificity  
267 (morphology) of the parasites. The 400 x g concentration speed produced comparable yield of  
268 parasites to 200 x g with no loss in parasite morphology. The concentrated pellet produced from a 400 x  
269 g concentration was also advantageous, as Parasep® tubes concentrate the entire collected specimen at  
270 once, leaving no residual stool for subsequent concentrations. The increased pellet yield negates this  
271 limitation by providing additional biomass in order to make multiple trichrome stains and wet mount  
272 preparations as may be needed without affecting sensitivity. This increased pellet yield is also valuable  
273 for larger parasitology laboratories that utilize residual specimens for teaching.

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

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

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

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

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

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

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

342 A significant advantage of Parasep® tubes is the increased productivity that can be achieved by  
343 the shorter time for specimen preparation (9). The workflow study performed in this investigation  
344 showed time savings of over half an hour for thirty specimens, and seventeen minutes for ten  
345 specimens. The majority of the time savings are gained in not having to transfer specimens to  
346 subsequent filters, not having to print and label multiple independent tubes/filters in order to maintain  
347 secure patient identification, and reducing the centrifugation time by 80% (or 8 minutes). For  
348 laboratories processing moderate-to-high volumes of O&P tests, this represents a significant advantage  
349 over the current laborious concentration procedures used in many laboratories. In fact, for a batch of  
350 30 specimens, the hands-on time savings were over 30 minutes. Given the centralization of O&P  
351 examinations in many reference and regional laboratories, the Parasep® tubes could provide significant  
352 benefit to laboratories that have experienced increasing volumes of these tests. A future area of  
353 exploration underway in our laboratory is automating the tube disassembly during processing, in an



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

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

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

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

378

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387

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413 "Parasep filter fecal concentrator tubes" for the detection of intestinal parasites in stool samples  
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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+ <i>B. hominis</i>	Uninterpretable
	200	1+ <i>B. hominis</i>	Uninterpretable
	400	1+ <i>B. hominis</i>	Uninterpretable
2	Unconcentrated	2+ <i>Chilomastix</i>	1+ <i>Chilomastix</i>
	200	1+ <i>Chilomastix</i>	2+ <i>Chilomastix</i>
	400	1+ <i>Chilomastix</i>	3+ <i>Chilomastix</i>
3	Unconcentrated	1+ <i>B. hominis</i> rare trophozoites	1+ <i>B. hominis</i>
	200	1+ <i>B. hominis</i> 1+ <i>E. nana</i>	1+ <i>B. hominis</i>
	400	1+ <i>B. hominis</i> 1+ <i>E. nana</i>	1+ <i>B. hominis</i>
4	Unconcentrated	1+ <i>B. hominis</i>	Negative
	200	1+ <i>B. hominis</i>	Negative
	400	1+ <i>B. hominis</i>	Negative
5	Unconcentrated	1+ <i>B. hominis</i>	1+ <i>B. hominis</i>
	200	1+ <i>B. hominis</i>	1+ <i>B. hominis</i>
	400	1+ <i>B. hominis</i>	1+ <i>B. hominis</i>
6	Unconcentrated	1+ trophozoites	Rare <i>E. histolytica/dispar</i>
	200	1+ trophozoites	1+ <i>E. histolytica/dispar</i>
	400	1+ trophozoites	1+ <i>E. histolytica/dispar</i>
7	Unconcentrated	1+ <i>B. hominis</i>	Rare <i>B. hominis</i>
	200	1+ <i>B. hominis</i>	Rare <i>B. hominis</i>
	400	1+ <i>B. hominis</i>	1+ <i>B. hominis</i>
8*	Unconcentrated	2+ <i>B. hominis</i> 1+ trophozoites	1+ <i>B. hominis</i> Rare <i>E. coli</i>
	200	3+ <i>B. hominis</i> 1+ trophozoites	1+ <i>B. hominis</i> 1+ <i>E. coli</i>
	400	2+ <i>B. hominis</i> 1+ trophozoites	1+ <i>B. hominis</i> 1+ <i>E. coli</i>
9**	Unconcentrated	4+ <i>B. hominis</i> 1+ trophozoites	1+ <i>B. hominis</i> 1+ <i>E. coli</i>
	200	3+ <i>B. hominis</i> 1+ trophozoites	1+ <i>B. hominis</i> 1+ <i>E. coli</i>
	400	2+ <i>B. hominis</i> 1+ trophozoites	1+ <i>B. hominis</i> 1+ <i>E. coli</i>
10	Unconcentrated	1+ <i>Giardia</i> cysts	Rare <i>Giardia</i>

419

200  
400

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

1+ *Giardia*  
1+ *Giardia*

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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+ <i>B. hominis</i>	1+ <i>B. hominis</i>
F2	<i>E. coli</i>	<i>E. coli</i>
F3	Negative	Negative
F4	1+ <i>B. hominis</i>	1+ <i>B. hominis</i>
F5	1+ <i>B. hominis</i> Dientamoeba fragilis	1+ <i>B. hominis</i> Dientamoeba fragilis
F6	Negative	Negative
F7	Negative	Negative
F8	<i>D. fragilis</i>	<i>D. fragilis</i>
F9	<i>E. coli</i>	<i>E. coli</i>
F10	2+ <i>B. hominis</i>	2+ <i>B. hominis</i>
F11	<i>E. nana</i> 2+ WBC	<i>E. nana</i> 2+ WBC
F12	3+ <i>B. hominis</i>	3+ <i>B. hominis</i>
F13	2+ <i>B. hominis</i>	2+ <i>B. hominis</i>
F14	2+ <i>B. hominis</i> <i>E. nana</i>	2+ <i>B. hominis</i> <i>E. nana</i>
F15	<i>Strongyloides rhabditoid</i> larvae	<i>Strongyloides rhabditoid</i> larvae
F16	<i>D. fragilis</i>	<i>D. fragilis</i>
F17	<i>E. nana</i>	<i>E. nana</i>
F18	2+ <i>B. hominis</i>	2+ <i>B. hominis</i> <u><i>E. coli</i></u>
F21	<i>Giardia</i>	<i>Giardia</i>
F22	1+ <i>B. hominis</i> <i>E. nana</i>	1+ <i>B. hominis</i> <i>E. nana</i>
F23	<i>D. fragilis</i>	<i>D. fragilis</i>
F24	<i>Giardia</i> 3+ WBC	<i>Giardia</i> 4+WBC
F25	1+ <i>B. hominis</i>	1+ <i>B. hominis</i>
F26	1+ <i>B. hominis</i> <i>E. nana</i>	1+ <i>B. hominis</i> <i>E. nana</i>
F27	1+ <i>B. hominis</i> <i>E. nana</i>	1+ <i>B. hominis</i> <i>E. nana</i>
F28	1+ <i>B. hominis</i> <i>E. nana</i>	1+ <i>B. hominis</i> <i>E. nana</i>
F29	1+ <i>B. hominis</i> <i>E. nana</i>	1+ <i>B. hominis</i> <i>E. nana</i>

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

423 WBC = white blood cell



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

PVA/Formalin Processing steps	30 run-Formalin/PVA		10 run-Formalin/PVA	
	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
<b>Build Run - Final time</b>	<b>0:51:46</b>	0:04:25	<b>0:27:41</b>	0:01:58

Parasep® Processing steps	30 run-Parasep		10 run-Parasep	
	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
<b>Build run - Final time</b>	<b>0:19:45</b>	0:01:52	<b>0:10:02</b>	0:00:44

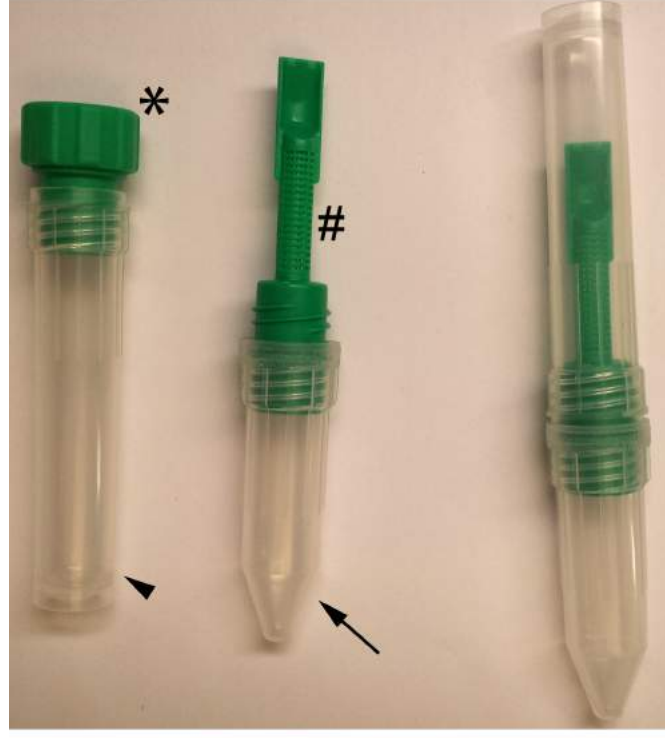
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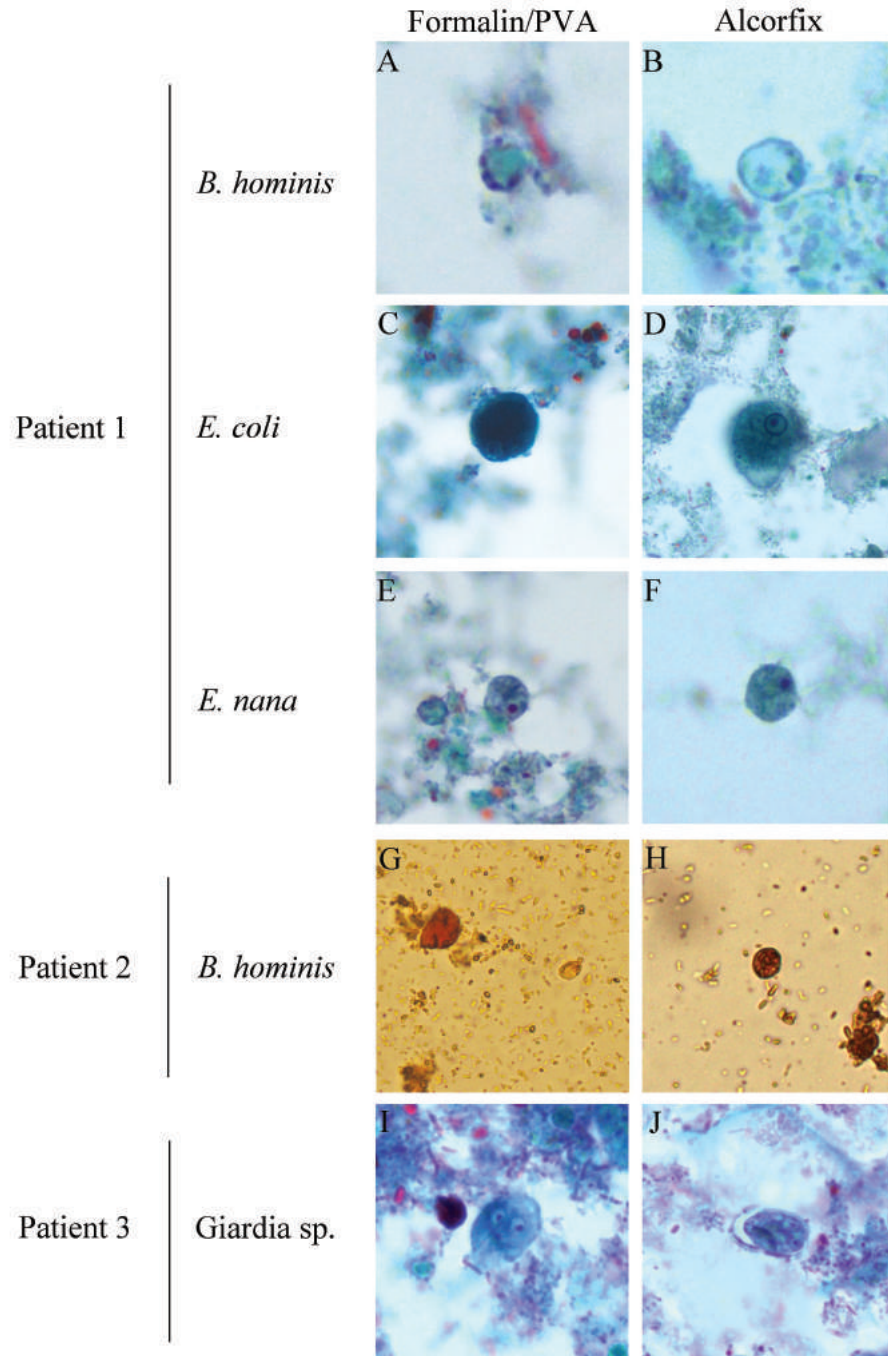
426 FIGURE TITLES:

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

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

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

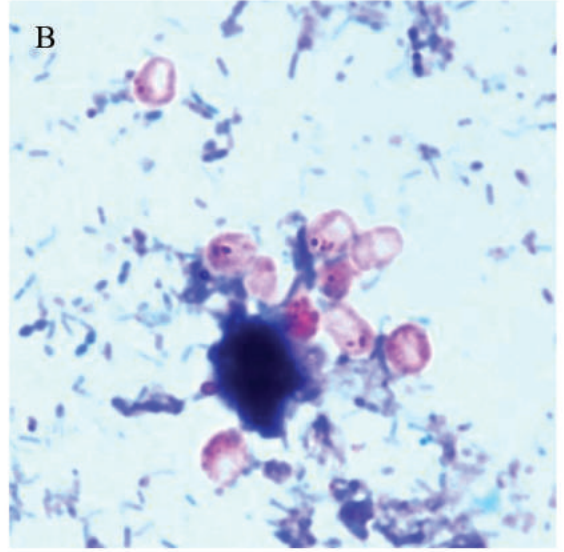
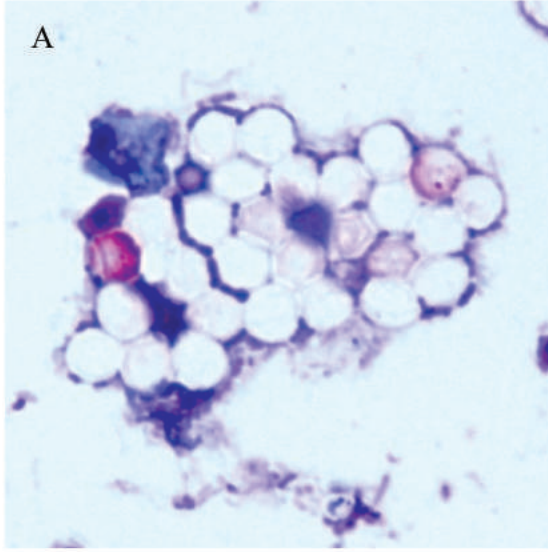




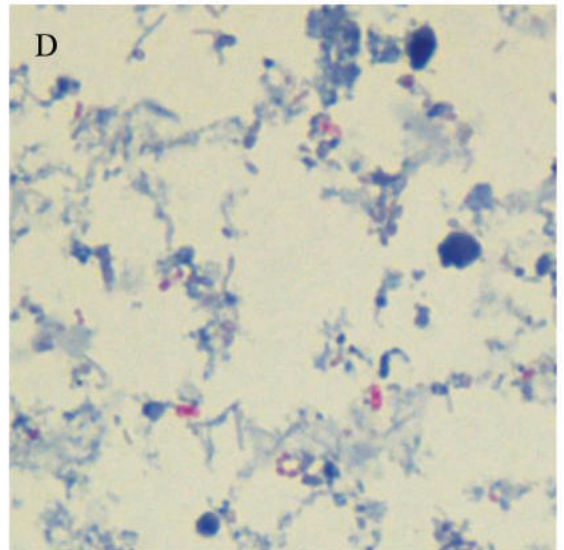
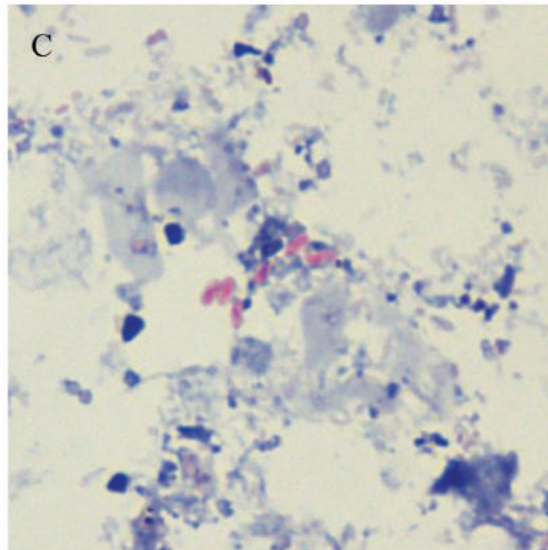
Formalin

Alcorfix

*C. parvum*



*E. intestinalis*



*E. cuniculi*

