

1 **Title:**

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3 EVALUATION OF ANATOMICALLY-DESIGNED FLOCKED RECTAL SWABS FOR THE  
4 MOLECULAR DETECTION OF ENTERIC PATHOGENS IN CHILDREN ADMITTED TO  
5 HOSPITAL WITH SEVERE GASTROENTERITIS IN BOTSWANA

6

7 **Running Title:**

8 Flocked rectal swabs for diarrheal pathogen detection

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38 **Abstract**

39

40 Two-hundred and eighty matched bulk stool and anatomically-designed flocced rectal swab  
41 samples were collected from children admitted to hospital with acute diarrhea in Botswana.  
42 Parents were asked about the acceptability of the swab collection method as compared with bulk  
43 stool sampling. All samples underwent identical testing with a validated 15 target (9 bacterial, 3  
44 viral, 3 parasite) commercial multiplex PCR assay. Flocced swabs had 12% higher yield for  
45 bacterial pathogen targets (241 vs. 212;  $p=0.003$ ) when compared with stool samples and similar  
46 yields for viral targets (110 vs. 113;  $p= 0.701$ ) and parasite targets (59 vs. 65;  $p= 0.345$ ). One  
47 hundred and sixty-four of the flocced swab – stool pairs were also tested with separate  
48 laboratory-developed bacterial and viral multiplex assays, and the flocced rectal swabs showed  
49 performance that was similar to that seen with commercial assay testing. Almost all  
50 parents/guardians found the swabs acceptable. Flocced rectal swabs significantly facilitate the  
51 molecular diagnosis of diarrheal disease in children.

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53 Key words:

54 Diarrhea; Shigella; Salmonella; Campylobacter; Enterotoxigenic E. coli; Rotavirus; Norovirus;  
55 Cryptosporidium; Entamoeba histolytica; flocced swab.

56 **Introduction**

57 Diarrheal disease remains a leading cause of global childhood morbidity and mortality, yet  
58 access to diagnostic laboratory testing is rarely available in much of the world. One of the  
59 barriers to diagnosing diarrheal disease, either for clinical or for surveillance purposes, is the  
60 difficulty and time delays in obtaining and transporting a bulk stool specimen. Several  
61 investigators have sought to overcome this barrier through the use of rectal swab specimens for  
62 culture, molecular and antigen testing, with variable results (1-5). Flocked swabs designed for  
63 respiratory and genitourinary sampling have been shown previously to acquire better samples  
64 when compared with more traditional fiber spun swabs (6,7). We used a specially designed  
65 flocked rectal swab (FLOQswab™ Copan Italia, Brescia, Italy) developed specifically for the  
66 diagnosis of diarrheal disease in children (Fig. 1) and then compared matched flocked rectal  
67 swabs to bulk stool samples in a clinical setting. Samples were collected from children admitted  
68 to hospital in Botswana with severe acute gastroenteritis and tested using an FDA cleared  
69 commercial multiplex PCR assay in order to assess performance across a broad number of  
70 bacterial, viral, and parasitic pathogens.

71 **Methods**

72 Children younger than 13 years of age, who were admitted to hospital with a diagnosis of acute  
73 gastroenteritis, were enrolled prospectively at the Princess Marina Hospital in Gaborone,  
74 Botswana. Princess Marina Hospital is the largest referral hospital in Botswana.  
75 Clinical data were collected and both the pediatric flocked rectal swab and bulk stool samples  
76 were obtained from each child as soon as possible after enrolment. Swab and stool samples were  
77 collected simultaneously if possible; otherwise, bulk stool was collected as soon as possible after  
78 rectal swab collection. Stool samples were collected and transported in sterile containers kept in

79 cooler boxes containing ice packs and then stored within 6 hours of collection at -80 °C. Parents  
80 or guardians of children who had both swab and stool specimens collected were asked about the  
81 acceptability of rectal swab specimen collection as compared with bulk stool collection using a  
82 5-point Likert scale. Parents or guardians gave signed consent, and the research protocol was  
83 approved by ethics committees at the University of Botswana, Botswana Ministry of Health,  
84 Princess Marina Hospital, University of Pennsylvania, and McMaster University, Hamilton,  
85 Canada.

86 Specimens were stored at -80 °C in dry swab tubes/cryovials prior to shipment on dry ice to  
87 McMaster University for testing. All matched swab – stool pairs underwent identical processing  
88 at the same time and by the same technologist. Pre analytical processing methods are shown in  
89 Table 1. There was a transition to the easyMAG™ extraction for samples collected after Jan. 29,  
90 2013 as this platform became available at the Botswana laboratory and establishment of on site  
91 validation and testing was planned. 10 ul aliquots of  $1.0 \times 10^9$  pfu/mL MS2 bacteriophage  
92 (Luminex Molecular Diagnostics, Cat. Number 0820002) and  $6 \times 10^8$  CFU/mL  
93 *Agrobacterium tumefaciens* (ATCC Cat. Number 33970) were added prior to pre-treatment as  
94 RNA and DNA internal positive controls, respectively. Reverse transcription, amplification and  
95 detection of 15 pathogen targets (3 viruses, 3 parasites and 9 bacteria) was performed using the  
96 Gastrointestinal Pathogen Panel (GPP) assay (Luminex Molecular Diagnostics, Toronto,  
97 Canada) on the MAGPIX™ system as per manufacturer instructions. The GPP assay has  
98 previously been evaluated (8,9) and will simultaneously detect the following pathogen analyte  
99 specific reagents (ASRs): Giardia, Cryptosporidium, *E. histolytica*, *Y. enterocolitica*, Salmonella,  
100 *E. coli* ST, *E. coli* LT, Shigella, *C. difficile* toxin A, *C. difficile* toxin B, Campylobacter, Vibrio  
101 cholerae, *E. coli* O157, Shiga Toxin 1, Shiga Toxin 2, Norovirus GI, Norovirus GII, Rotavirus A,

102 and Adenovirus 40/41. Samples collected from Sept. 6, 2012 until Jan. 29, 2013 (n=164) were  
103 also tested in parallel with two laboratory developed multiplex PCR assays, one targeting the  
104 three most prevalent bacterial pathogens (Salmonella spp., Shigella spp., Campylobacter  
105 jejuni/coli) (10-12) and the other targeting rotavirus A, norovirus GI/GII, and all adenovirus (13-  
106 16). These assays were adapted from the literature and primer and probe sequences are listed in  
107 Table 2. Five ul of extracted nucleic acid from match stool and swab samples (processed as  
108 described above) were added to the primers, probes and mastermix reagents; QuantiTect  
109 Multiplex No Rox PCR Kit (Qiagen, Mississauga, Canada, Cat. Number 204743) for the  
110 bacterial multiplex and QuantiTec Multiplex PCR Kit (Qiagen, Mississauga, Canada, Cat.  
111 Number 204543) for the viral multiplex. Statistical analysis was performed using STATA  
112 statistical analysis software version 11 (STATA Corp., College Station, TX). McNemar's test  
113 for paired samples was used to assess swab vs. bulk stool detection of target pathogens.  
114 Bacterial, viral and parasitic targets were each analyzed separately. Sensitivity for each  
115 pathogen ASR was calculated using the reference standard of the presence of the ASR in either  
116 sample. Paired t test was used to compare Ct (threshold cycle) values in matched positive  
117 samples tested by the LDT assays.

118

## 119 **Results**

120 Specimens were collected from September 6, 2012 until Aug 16, 2013. A total of 338 flocked  
121 rectal swab specimens were collected of which 280 (83%) also had matched bulk stool collected.  
122 Parents or guardians of 279 of the 280 subjects answered the questionnaire regarding the  
123 acceptability of rectal swab sampling as follows: 266 (95%) responded "acceptable", 8  
124 responded "slightly acceptable", 3 responded "neutral", none responded "slightly unacceptable",

125 and 2 responded “unacceptable”. Only one sample showed frank inhibition of MS2 (bulk stool  
126 sample) but no samples showed inhibition of *A. tumefaciens* DNA detection. One child did not  
127 have a rectal swab collected due to imperforate anus and one child’s guardian refused the rectal  
128 swab collection. The median time from swab collection to bulk stool collection was 5 minutes  
129 longer than for a swab sample (interquartile range: 0.03 hours, 2.7 hours). Comparison of GPP  
130 and LDT assay results on 164 stool samples revealed relatively close concordance between  
131 assays for 3 bacterial and 2 viral targets (see Table 3). Adenovirus was not compared given that  
132 GPP targets serotypes 40/41 while the LDT targets all adenoviruses. Matched swab-stool pair  
133 GPP testing results are shown in Table 4. *Y. enterocolitica* and *Vibrio cholerae* targets were not  
134 detected in any sample. Assessing the sum total of all pathogen targets, the flocced swab samples  
135 detected a total of 410 targets and the bulk stool samples yielded 390 pathogen targets ( $p =$   
136  $0.113$ ). Among the 280 flocced swab samples tested with the GPP assay, 110 had a single  
137 pathogen detected; 73, 33, 10, and 3 had two, three, four or five pathogens, respectively; and 51  
138 had no pathogens detected. Among the 280 stool samples, 113 had a single pathogen detected;  
139 63, 35, 9, and 2 had two, three, four or five pathogens, respectively; and 58 stool samples had no  
140 pathogens detected. There was an average of 1.46 pathogens detected per patient for swab  
141 samples and 1.39 pathogens per patient with stool sample testing. Swab samples detected 12%  
142 more bacterial targets when compared to matched stool sample testing (241 vs. 212;  $p = 0.003$ ).  
143 There was no significant difference in detection of viral pathogens (110 vs. 113;  $p = 0.701$ ) or  
144 protozoal pathogens (59 vs. 65;  $p = 0.345$ ). Focusing on the pathogens for which antimicrobial  
145 treatment is generally recommended in the context of severe gastroenteritis requiring admission  
146 to hospital (*Shigella*, *Campylobacter*, ETEC, *Cryptosporidium*, *Giardia*, and *E. histolytica*),  
147 flocced swab samples identified 226 pathogens and stool samples identified 203 pathogens ( $p =$

148 0.009, McNemar test). Testing results from the laboratory-developed multiplex PCR assays on  
149 164 matched flocced swab-stool pairs are shown in Table 5. There was a total of 189 pathogens  
150 targets detected in the flocced swab samples and 167 detected in the matched stool specimens. Ct  
151 (threshold cycle) values for matched concordant positive swab and stool samples are shown in  
152 Table 6. Ct values were similar for *Shigella*, *Salmonella* and adenovirus. Ct values were lower in  
153 swab samples concordant for *Campylobacter* and higher for swab samples concordant for  
154 norovirus.

155

## 156 **Discussion**

157 We found that samples collected using specifically designed flocced rectal swabs from children  
158 admitted with severe acute gastroenteritis in Botswana allowed for significantly higher bacterial  
159 pathogen detection when using multiplex PCR assays as compared to the same testing on  
160 matched bulk stool samples. Our population had a high prevalence of pathogens detected via  
161 molecular multiplex assays, which is in keeping with other studies done of pediatric  
162 gastroenteritis in developing country settings (17).

163 As outlined in the methods section, during the study we changed our extraction method from the  
164 QIA Symphony™ platform to the easyMAG™ platform, as the latter had become available at the  
165 laboratory in Botswana and we planned to transition testing on site using this method. Thirty-  
166 seven paired bulk stool/swab samples were processed using both extraction platforms, and there  
167 was no clear difference in results (data not shown). easyMAG™ extraction using the Belkin  
168 ceramic beads did detect three additional *E. histolytica* positive flocced swab samples which  
169 were not detected using the glass bead lysis. As shown in Table 3, we also found 5 additional *E.*  
170 *histolytica* positives - all detected in flocced swab samples and all in samples processed using the



171 latter pre-analytical method using ceramic bead lysis with easyMAG™ extraction. Although not  
172 proven, we surmise that the additional positives were a result of better cyst lysis with ceramic  
173 beads, but this requires further study. However, this potential difference in *E. histolytica*  
174 detection by pre-analytic method is unlikely to affect the results of the swab and bulk stool  
175 comparison, as all matching samples were process identically, and neither viral nor bacterial  
176 target amplification was affected by extraction methods.

177

178 A similar study carried out with children presenting with diarrhea in Rwanda compared regular  
179 flocked swabs to bulk stool PCR and found similar yields for qualitative detection of multiple  
180 bacterial, viral, and parasitic pathogens (18). Our swab showed similar detection for most  
181 pathogen targets but actually had higher yield for several bacterial targets. There are several  
182 plausible explanations for this finding. The rectal FLOQSwab™ was specially designed such that  
183 it would sample just beyond the anal canal at the columnar epithelium. The swab has a  
184 lengthened flocked surface and a “stopper” at the 3.2 cm mark which extends just proximal to  
185 the surgical anus of most children under 3 years of age (19). Many of the bacterial and some  
186 protozoal pathogens of diagnostic interest reside in this anatomic location and therefore we  
187 hypothesized that this would be the ideal location to sample. Conversely, bulk stool samples  
188 contain more contents derived largely from the small intestine which may in fact dilute cellular  
189 material of interest contained in the colonic mucosal surface. Given that we used molecular  
190 diagnostics, another potential explanation is that bulk stool samples contain more inhibitory  
191 material than flocked swab samples. We included MS2 phage and *Agrobacterium tumefaciens* as  
192 internal RNA and DNA controls, respectively, and found frank RNA inhibition with only one

193 bulk stool sample. However, these controls may not rule out relative inhibition, which may have  
194 affected bulk stool to a greater extent than rectal swabs.

195 Our group and others have found relatively high rates of mortality in children in sub-Saharan  
196 Africa presenting with moderate to severe acute gastroenteritis (20,21). Many of these children  
197 who die from gastroenteritis are found to have treatable enteropathogens detected in their stool  
198 (20, 22). Given that in our study we were not able to collect a matching bulk stool sample from  
199 17% of enrolled children prior to discharge or death, and that for an additional 25% of children it  
200 took  $\geq 2.7$  hours to collect a matching stool sample, our data suggest that point of care  
201 diagnostics using bulk stool samples would be a challenge for a large proportion of children even  
202 in the in patient setting. The combination of rapid sample acquisition with sensitive rapid  
203 detection methods such as PCR may allow for targeted treatment and the potential for  
204 significantly improved outcomes for this common and in many places often deadly infection.

205

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218

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220 Daytona Beach, United States, April 28 – May 1st, 2013 and at the Annual Pediatric  
221 Academic Society Meeting, Vancouver, Canada, May 5<sup>th</sup>, 2014.

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**Figure 1 Pediatric rectal enteropathogen flocced swab**



**Table 1. Pre-analytic processing methods used in the study**

Matched samples collected from Sept. 6, 2012 until Jan. 29, 2013 (n=164)	Matched samples collected from Jan. 30, 2013 until Aug. 16, 2013 (n=116)
1. Swabs were eluted in 1 ml of eNAT™ (Copan Italia, Brescia, Italy) and 100 µl of the matching stool sample was added to 900 µl of eNAT™, both tubes contained 1 mm glass beads (Biospec Products, Bartlesville, OK)	1. Swabs were eluted in 1 ml of easyMAG™ lysis buffer (BioMerieux, Durham, NC) and 100µl of the matching stool sample was added to 900 µl of easyMAG™ lysis buffer, both tubes containing Bertin SK38 Soil Mix beads (BioAmerica Inc., Miami, FL)
2. 5 minutes of lysis via vortex mixing and then held for 10 minutes at room temperature, followed by centrifugation at 14 000 rpm for 2 minutes	2. 5 minutes of lysis via vortex mixing and then held for 10 minutes at room temperature, followed by centrifugation at 14 000 rpm for 2 minutes
3. Crude lysates were stored at -80 °C until nucleic acid extraction performed using 200 ul of cleared supernatant via QIASymphony™ (Qiagen, Germantown USA) using the DSP Virus/Pathogen Mini-Kit with an elution volume of 70 ul	3. Crude lysates were stored at -80 °C until nucleic acid extraction performed using 200 ul of cleared supernatant via Nuclisense easyMAG™ using extraction protocol Specific B and an elution volume of 70 ul

**Table 2. Pathogen primers and probes used in the laboratory developed multiplex assays**

Pathogen Target	Forward primer	Reverse primer	Probe Sequence	Target	Reference
Salmonella	CTCACCAGGAGATTACAACATGG	AGCTCAGACCAAAAGTGACCATC	CAC CGA CGG CGA GAC CGA CTT T	ttr gene	10
Shigella	CCTTTCCGGGTTCCCTGA	CGGAATCCGGAGGTATTGC	CGC CTT TCC GAT ACC GTC TCT GCA	ipaH gene	11
Campylobacter	CTGCTTAACACAAGTTGAGTAGG	TTCCTTAGGTACCGTCAGAA	TGTCATCCTCCACGCGGCTTGCTGC	16SrRNA	12
Rotavirus A	GGAKGYCTGTA CTM TTG TCA	CCAGTTTGRAASTCATTTC	GAATATAAT/ZEN/GTACCTTCRACAATTTTGTCTAGCATC	VP6 gene	13
Norovirus GI	CGYTGATGCGNTTYCATGA	CTTAGACGCCATCATCATTYAC	AGATYGCGRTCYCCTGTCCA	RNA Polymerase/Capsid	14,15
Norovirus GII	CARGARBCNATGTTYAGRTGGATGAG	TCGACGCCATCTTCATTCACA	TGGGAGGGCGATCGCAATCT	RNA Polymerase/Capsid	14,15
Adenovirus	CAGGACGCCTCGGRGTAYCTSAG	GGAGCCACVGTGGGRTT	CCGGTCTGGTGCA GTTTGCCCGC	Hexon	16

Table 3. Comparison of performance of Gastrointestinal Pathogen Panel (GPP™) assay and Laboratory developed Test (LDT) performance on shared bacterial and viral targets using 164 bulk stool samples

	Both positive	GPP (+) only	LDT (+) only	Both negative	Concordance	McNemar <i>P</i>
<b>Shigella</b>	42	0	2	120	98.8%	0.50
<b>Campylobacter</b>	23	1	7	133	95.1%	0.07
<b>Salmonella</b>	12	1	3	148	97.6%	0.62
<b>Rotavirus</b>	13	0	0	151	100%	1.00
<b>Norovirus GI/GII</b>	28	0	4	132	97.6%	0.12

**Table 4. Comparison of pathogen target detection in 280 matched bulk stool and flocked swab sample pairs using xTAG GPP™ assay**

	Either Sample Positive*	Both Positive	Swab (+) Only	Stool (+) Only	Both Negative	Swab Sensitivity	Stool Sensitivity	McNemar <i>P</i>
Shigella	82	63	16	3	198	96.3%	80.5%	<0.01
Campylobacter	56	43	11	2	224	96.6%	80.4%	0.02
Salmonella	52	29	13	10	228	80.8%	75%	0.68
ETEC LT/ST	36	25	9	2	244	94.4%	74.3%	0.06
E. coli O157	10	7	2	3	268	75.0%	83.3%	1.00
STEC stx1/stx2	11	6	2	3	269	72.7%	81.8%	1.00
C. diff toxin A/B	23	8	7	8	257	65.2%	69.6%	1.00
All bacterial combined	272	181	60	31	1688	88.6%	77.9%	<0.01
Norovirus GI/GII	58	41	5	12	222	79.3%	91.4%	0.14
Rotavirus A	32	29	2	1	248	96.9%	93.8%	1.00
Adenovirus 40/41	35	28	5	2	245	94.3%	85.7%	0.45
All viral combined	125	98	12	15	715	88.0%	90.4%	0.70
Cryptosporidium	47	33	6	8	233	83.0%	87.2%	0.79
Giardia	24	15	0	9	256	62.5%	100%	<0.01
E. histolytica	5	0	5	0	275	100%	0%	0.06
All protozoa combined	76	48	11	17	764	77.6%	85.5%	0.34

\* Either sample positive is set as the reference for calculation of sensitivity

Table 5. Pathogen target detection in 164 matched bulk stool and flocked swab sample pairs using laboratory developed multiplex assays

	<b>Either Sample Positive*</b>	<b>Both Samples Positive</b>	<b>Swab (+) only</b>	<b>Stool (+) only</b>	<b>Both Samples Negative</b>	<b>Swab Sensitivity</b>	<b>Stool Sensitivity</b>	<b>McNemar <i>P</i></b>
<b>Shigella</b>	<b>54</b>	<b>42</b>	<b>12</b>	<b>0</b>	<b>110</b>	<b>100%</b>	<b>77.8%</b>	<b>&lt;0.01</b>
<b>Campylobacter</b>	<b>43</b>	<b>27</b>	<b>13</b>	<b>3</b>	<b>121</b>	<b>93.0%</b>	<b>69.8%</b>	<b>0.02</b>
<b>Salmonella</b>	<b>29</b>	<b>11</b>	<b>13</b>	<b>5</b>	<b>135</b>	<b>82.8%</b>	<b>55.2%</b>	<b>0.10</b>
<b>All bacterial combined</b>	<b>126</b>	<b>80</b>	<b>38</b>	<b>8</b>	<b>366</b>	<b>93.6%</b>	<b>69.8%</b>	<b>&lt;0.01</b>
<b>Norovirus GI/GII</b>	<b>32</b>	<b>25</b>	<b>0</b>	<b>7</b>	<b>132</b>	<b>78.1%</b>	<b>100%</b>	<b>0.02</b>
<b>Rotavirus A</b>	<b>13</b>	<b>12</b>	<b>0</b>	<b>1</b>	<b>151</b>	<b>92.3%</b>	<b>100%</b>	<b>1.0</b>
<b>Adenovirus</b>	<b>36</b>	<b>32</b>	<b>2</b>	<b>2</b>	<b>128</b>	<b>94.4%</b>	<b>94.4%</b>	<b>1.0</b>
<b>All viral combined</b>	<b>81</b>	<b>69</b>	<b>2</b>	<b>10</b>	<b>411</b>	<b>87.7%</b>	<b>97.5%</b>	<b>0.04</b>

\* Either sample positive is set as the reference for calculation of sensitivity

**Table 6. Mean Threshold cycle (Ct) values for matched positive samples and Ct value differences by laboratory developed real-time PCR**

Pathogen Target	Rectal Swab	Stool Sample	Difference in Ct values (95% CI)	p value by paired t-test
Shigella (n=42)	25.96	26.34	-0.39 (-1.69 to 0.91)	0.55
Salmonella (n=11)	33.69	34.88	-1.19 (-3.5 to 1.14)	0.28
Campylobacter (n=27)	27.98	30.33	-2.35 (-4.05 to -0.064)	<0.01
Rotavirus (n=12)	26.86	25.60	1.27 (-1.33 to 3.86)	0.31
Adenovirus (n=32)	28.01	27.42	0.60 (-0.98 to 2.17)	0.20
Norovirus GI/GII (n=25)	27.82	23.79	4.03 (2.18 to 5.89)	<0.01

Ct = Threshold cycle, CI = Confidence Interval