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Application of Alternative Nucleic Acid Extraction Protocols to ProGastro SSCS Assay for Detection of Bacterial Enteric Pathogens

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As an alternative to automated extraction, fecal specimens were processed by investigational lysis/heating (i.e., manual) and by chromatography/centrifugation (i.e., column) methods. ProGastro SSC and Shiga toxin-producing *Escherichia coli* (i.e., STEC) indeterminate rates for 101 specimens were 1.0% to 3.0% for automated, 11.9% for manual, and 24.8% to 37.6% for column methods. Following freeze-thaw of 247 specimens, indeterminate rates were 1.6% to 2.4% for manual and 0.8 to 5.3% for column methods. Mean processing times for manual and column methods were 30.5 and 69.2 min, respectively. Concordance of investigational methods with automated extraction was ≥98.8%.

Accurate and rapid laboratory diagnostics for patients with gastroenteritis contribute to clinical management and expedient public health notification (1). Molecular-based assays have demonstrated reliable identification of common bacterial agents of gastrointestinal disease (2–6), yet performance of these assays may present obstacles for laboratories not possessing automated means of nucleic acid extraction and workflow challenges for other laboratories. Development of alternative processing protocols must ensure mitigation of inhibitory agents endogenous to fecal specimens (7, 8) and of those produced by commensal enteric Gram-negative bacteria (9).

ProGastro SSCS (Hologic, San Diego, CA) is an FDA-cleared multiplex real-time PCR assay for qualitative detection of nucleic acid specific to *Salmonella* spp., *Shigella* spp., *Campylobacter coli*/*Campylobacter jejuni* (undifferentiated), and Shiga toxin-producing *Escherichia coli* (STEC). The assay is indicated on preserved fecal specimens from symptomatic patients with signs and symptoms of gastroenteritis. Furthermore, performance of PCR is to be preceded by nucleic acid extraction using either of two automated platforms (10). Compared to conventional culture/enzyme immunoassay modalities, and paired with a bidirectional sequencing adjudicator, the assay demonstrated 100% accuracy in detection of these nucleic acids from preserved fecal specimens (6). Valid results were procured at a rate of 98.8% using NucliSens easyMag system (bioMérieux) automated extraction.

One commercial molecular assay for another gastrointestinal pathogen, *Clostridium difficile*, utilizes facets of manual extraction paradigms described by Boom et al. (11). Importantly, glass particles were found to bind nucleic acids in the context of crude cellular lysates. Previous data describe efficient manual processing protocols for molecular detection of toxigenic *C. difficile* with rapid turnaround time (12). Moreover, quality assurance data revealed a 0.51% molecular *C. difficile* indeterminate rate (indicative of insufficient internal control amplification) from 10,131 primary specimens tested over 3 years (Wheaton Franciscan Laboratory, unpublished findings). The objective of this study was to investigate application of off-label, nonautomated extraction protocols, including methods previously described by Boom et al. (11), to clinical performance of ProGastro SSCS. (Results of this work were presented in part at the 115th General Meeting of the American Society for Microbiology, New Orleans, LA, 30 May to 2 June 2015.)

A Wheaton Franciscan healthcare institutional review board–approved study protocol is outlined in Figure 1. Primary diarrheal specimens submitted as part of routine care were preserved in Cary Blair transport medium (Meridian Biosciences, Cincinnati, OH). NucliSens easyMag extractions (referred to as automated method) were performed by an outside laboratory per package insert instructions, beginning with 50 μl of preserved stool diluted in 450 μl of Cary Blair transport medium plus 10 μl of additive ProGastro SSCS-provided internal control nucleic acid. Alternatively, QIAamp fast DNA stool minikit (Qiagen, Alameda, CA) extractions (referred to as column method) were prepared per manufacturer guidelines for pathogen detection (13) using approximately 180 mg of preserved stool with 1.0 ml of InhibitEx buffer and 10 μl of additive internal control nucleic acid. Nucleic acid extracts from these two protocols were frozen at −70°C prior to PCR analysis. In addition, specimens were subjected to a lysis- and glass bead-based preparation (referred to as manual method). In brief, 200 μl of preserved stool was diluted with 200 μl of Tris-HCl buffer (BD lysis kit; BD Diagnostics, Sainte-Foy, Quebec) in 2.0-ml polypropylene screw-cap microcentrifuge tubes (Corning Life Sciences, Corning, NY) and then was vortexed for 1 min. Then, 10 μl of the suspension was transferred to lysis tubes containing glass beads along with additive 40 μl of Tris-HCl buffer and 10 μl of internal control nucleic acid. Suspensions were vortexed for 5 min, pulse-centrifuged (14,800 rpm, 10 s), heated for 6 min at 95°C, and then frozen at −70°C. Equal-volume Cary Blair medium was substituted for preserved fecal specimen in one tube per extraction batch as a negative extraction control (referred to as Cary Blair aliquots) and subjugated to all aforementioned steps.

Following one freeze-thaw cycle, individual extracts and cellu-

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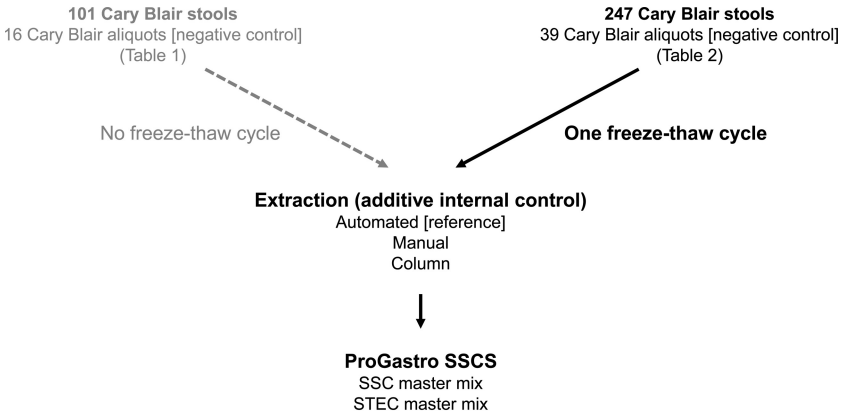

FIG 1 Study algorithm.

lar lysates (pulse-centrifuged for 10 s) were incubated in both STEC (detection of internal control sequence and nucleic acids specific to *E. coli* Shiga toxin) and SSC (detection of internal control sequence plus nucleic acids specific to *Salmonella* spp., *Shigella* spp., and *C. coli*/*C. jejuni*) master mixes as components of ProGastro SSCS (10). Per package insert specifications, the maximum cycle threshold (C_T) value yielding a valid internal control nucleic acid amplification result on both SSC and STEC master mixes was 45.0.

Efficacy of extraction modalities for amplification of internal control was assessed in the context of 101 clinical specimens (referred to as Cary Blair stool) and 16 Cary Blair aliquots. Of the 702 total results generated by the three extraction modalities distributed among SSC and STEC master mixes, 124 (17.7%) were initially discordant, with 117 of these being indeterminate (Table 1). An unacceptable percentage of indeterminate SSC results was generated following manual (11.9%) and column (24.8%) extractions of Cary Blair stool ($P \leq 0.016$ versus automated). When the mean C_T values of specimens that did yield valid results for all three extraction modalities were compared, less internal control amplicon was generated by column ($P < 0.0001$ versus manual) and manual ($P < 0.0001$ versus automated) methods. Moreover, 75% of Cary Blair aliquots failed to generate a valid result via column extraction, with decreased internal control amplification (C_T, 40.05; $P \leq 0.013$ versus automated and manual). Similar findings resulted from incubation of automated, manual, and column extracts/lysates with the STEC master mix.

As studies have espoused the value of a freeze-thaw cycle for removal of endogenous inhibitors from primary specimens (14–16), extractions were subsequently processed following storage of 247 Cary Blair stools and 39 Cary Blair aliquots overnight at −70°C. The 101 previously assessed Cary Blair stools (Table 1) were a component of this study set. Of the 1,716 total results generated by the three extraction modalities distributed among SSC and STEC master mixes, just 58 (3.4%) were initially discordant, with 47 of these being indeterminate (Table 2). Valid Cary Blair stool results were generated from SSC master mix at a rate of ≥98.4% after automated, manual, and column extraction ($P \geq 0.41$). Both automated and manual extraction allowed for sufficient internal control amplification from Cary Blair stool and Cary Blair aliquots (C_T, ≤34.82; $P \geq 0.22$ for intermodality comparisons). An unacceptable indeterminate result rate from Cary Blair aliquots remained for column extraction (20.5%), while internal control amplification was less efficient (C_T, 39.67; $P < 0.0001$ versus automated and manual). Also, 5.3% and 28.2% of Cary Blair stool and Cary Blair aliquot STEC master mix results, respectively, derived from column extraction were indeterminate. Internal control amplification was less robust than automated and manual extraction ($P < 0.0001$). Manual extraction of clinical and control material exhibited similar performance to automated ex-

TABLE 1 Characterization of initial ProGastro SSCS results derived from three extraction modalities on 101 Cary Blair-preserved stool specimens and 16 Cary Blair aliquots without antecedent freeze-thaw cycle

Extraction modality	SSC master mix				STEC master mix			
	Cary Blair stool		Cary Blair aliquots (negative control)		Cary Blair stool		Cary Blair aliquots (negative control)	
	No. (%) of indeterminate results	Mean internal control C_T[a]	No. (%) of indeterminate results	Mean internal control C_T[a]	No. (%) of indeterminate results	Mean internal control C_T[a]	No. (%) of indeterminate results	Mean internal control C_T[a]
Automated	3 (3.0)	34.93	0 (0.0)	35.43	1 (1.0)	34.21	0 (0.0)	35.40[b]
Manual	12 (11.9)	36.55	0 (0.0)	35.60	12 (11.9)	35.90	1 (6.3)	35.00
Column	25 (24.8)	38.42	12 (75.0)	40.05	38 (37.6)	38.49	13 (81.3)	37.70

[a] Provided for specimens yielding detectable internal control amplification with all three extraction modalities.
[b] Differences between automated, manual, and column ($P \geq 0.54$).

TABLE 2 Characterization of initial ProGastro SSCS results derived from three extraction modalities on 247 Cary Blair-preserved stool specimens and 39 Cary Blair aliquots following a single freeze-thaw cycle

Extraction modality	SSC master mix				STEC master mix			
	Cary Blair stool		Cary Blair aliquots (negative control)		Cary Blair stool		Cary Blair aliquots (negative control)	
	No. (%) of indeterminate results	Mean internal control C_T[a]	No. (%) of indeterminate results	Mean internal control C_T[a]	No. (%) of indeterminate results	Mean internal control C_T[a]	No. (%) of indeterminate results	Mean internal control C_T[a]
Automated	2 (0.8)	34.57	0 (0.0)	34.82	1 (0.4)	34.05	0 (0.0)	35.06
Manual	4 (1.6)	34.34	0 (0.0)	34.81	6 (2.4)	33.58	0 (0.0)	34.31
Column	2 (0.8)	36.58	8 (20.5)	39.67	13 (5.3)	36.41	11 (28.2)	39.11

[a] Provided for specimens yielding detectable internal control amplification with all three extraction modalities.

traction ($P \geq 0.06$), with an increase in internal control amplification efficiency ($P \leq 0.03$).

Initially discordant results derived from extractions of freeze-thawed Cary Blair stool and Cary Blair aliquots were adjudicated by repeat PCR performance on the frozen extracts/lysates. Twelve of 47 indeterminate results could not be resolved ($n = 4$, manual extraction; $n = 8$, column extraction). Performance indices of manual and column modalities (≥91.7% sensitivity, ≥99.6% specificity) rivaled those of reference automated extraction in this study set that yielded 15 *Salmonella* spp., 12 *Shigella* spp., 16 *C. coli*/*C. jejuni*, and 8 Shiga toxin-producing *E. coli* by SSC and STEC master mixes (Table 3). While this investigation was not designed to examine the sensitivity of ProGastro SSCS compared to reference methods, such as culture and enzyme immunoassay (6), 36.4%, 16.7%, and 7.7% more instances of *C. coli*/*C. jejuni*, *Shigella* spp., and *Salmonella* spp., respectively, were detected via manual extraction than by culture (data not illustrated). Final concordance rates of manual and column methods with the automated reference were 98.8% and 99.2%, respectively.

In design of the manual protocol, the 1:10 dilution aligned with package insert specifications relative to automated extraction. Furthermore, Monteiro et al. (17) described the performance of *Helicobacter pylori* PCR using an initial stool dilution of 1:10. Amendment of the column protocol (performed at an approximate 1:7 dilution) to allow for an initial 1:10 Cary Blair stool or Cary Blair aliquot dilution may be warranted in further studies to determine if rates of initially indeterminate results can be decreased to the level of the manual method. In our evaluation, the initial dilution was accomplished with consecutive 1:2 and 1:5 dilutions to allow for a larger sampling of Cary Bair stool. Despite the increased labor, mean processing time for manual extraction of a 7-specimen batch (30.5 min) was less than both the column method (69.2 min; $P < 0.0001$) and the automated reference (60 min; independent laboratory, personal communication). These data do not consider the time necessary to execute the freeze-thaw cycle itself. However, these components can easily be managed by laboratory support personnel as the preserved specimen arrives in the laboratory and by a technologist as part of the routine testing workload.

Similar to other PCR (12, 18, 19) and reverse transcriptase PCR (14) assays, additional workflow optimization of ProGastro SSCS can be accomplished by utilization of batch-prepared frozen master mix tubes (20). In summary, exposure of Cary Blair stool and medium aliquots to a single freeze-thaw cycle accommodates off-label nucleic acid extraction for accurate performance of ProGastro SSCS. This modification, particularly in the context of an efficient lysis protocol, may expand opportunities for laboratories to contribute to the rapid diagnosis of bacterial gastroenteritis.

TABLE 3 Adjudicated performance indices of ProGastro SSCS derived from Cary Blair stool subjected to three extraction modalities following one freeze-thaw cycle

Performance index by extraction modality	Nucleic acid detection specific to:			
	Salmonella spp.	*Shigella* spp.	*Campylobacter coli*/*Campylobacter jejuni*	Shiga toxin
Sensitivity (%)				
Automated	100.0	100.0	100.0	100.0
Manual	100.0	91.7	93.8	100.0
Column	93.3	100.0	93.8	100.0
Specificity (%)				
Automated	100.0	100.0	100.0	100.0
Manual	100.0	99.6	100.0	100.0
Column	100.0	100.0	100.0	100.0

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REFERENCES

1. **Prakash VP, LeBlanc L, Alexander-Scott NE, Skidmore J, Simmons D, Quilliam D, Chapin KC.** 2015. Use of a culture-independent gastrointestinal multiplex PCR panel during a shigellosis outbreak: considerations for clinical laboratories and public health. J Clin Microbiol **53:**1048–1049. http://dx.doi.org/10.1128/JCM.03374-14.
2. **Knabl L, Grutsch I, Orth-Höller D.** 2016. Comparison of the BD Max enteric bacterial panel assay with conventional diagnostic procedures in diarrheal stool samples. Eur J Clin Microbiol Infect Dis **35:**131–136. http://dx.doi.org/10.1007/s10096-015-2517-4.
3. **Buss SN, Leber A, Chapin K, Fey PD, Bankowski MJ, Jones MK, Rogatcheva M, Kanack KJ, Bourzac KM.** 2015. Multicenter evaluation of the BioFire FilmArray gastrointestinal panel for etiologic diagnosis of infectious gastroenteritis. J Clin Microbiol **53:**915–925. http://dx.doi.org/10.1128/JCM.02674-14.
4. **Patel A, Navidad J, Bhattacharyya S.** 2014. Site-specific clinical evaluation of the Luminex xTag gastrointestinal pathogen panel for detection of infectious gastroenteritis in fecal specimens. J Clin Microbiol **52:**3068–3071. http://dx.doi.org/10.1128/JCM.01393-14.
5. **Khare R, Espy MJ, Cebelinski E, Boxrud D, Sloan LM, Cunningham SA, Pritt BS, Patel R, Binnicker MJ.** 2014. Comparative evaluation of two

commercial multiplex panels for detection of gastrointestinal pathogens by use of clinical stool specimens. J Clin Microbiol **52:**3667–3673. http://dx.doi.org/10.1128/JCM.01637-14.

6. **Buchan BW, Olson WJ, Pezewski M, Marcon MJ, Novicki T, Uphoff TS, Chandramohan L, Revell P, Ledeboer NA.** 2013. Clinical evaluation of a real-time PCR assay for identification of *Salmonella*, *Shigella*, *Campylobacter* (*Campylobacter jejuni* and *C. coli*), and Shiga toxin-producing *Escherichia coli* isolates in stool specimens. J Clin Microbiol **51:**4001–4007. http://dx.doi.org/10.1128/JCM.02056-13.
7. **Furukawa K, Bhavanandan VP.** 1983. Influences of anionic polysaccharides on DNA synthesis in isolated nuclei and by DNA polymerase alpha: correlation of observed effects with properties of the polysaccharides. Biochim Biophys Acta **740:**466–475. http://dx.doi.org/10.1016/0167-4781(83)90096-9.
8. **Greenfield L, White TJ.** 1993. Sample preparation methods, p 122–137. *In* Persing DH, Smith TF, Tenover FC, White TJ (ed), Diagnostic and molecular microbiology: principles and applications. American Society for Microbiology, Washington, DC.
9. **Chan JWYF, Goodwin PH.** 1995. Extraction of genomic DNA from extracellular polysaccharide-synthesizing Gram-negative bacteria. Biotechniques **18:**418–422.
10. **Prodesse**2014. ProGastro product insert. Hologic, Inc., San Diego, CA.
11. **Boom R, Sol CJ, Salimans MM, Jansen CL, Wertheim-van Dillen PM, van der Noordaa J.** 1990. Rapid and simple method for purification of nucleic acids. J Clin Microbiol **28:**495–503.
12. **Munson E, Bilbo D, Paul M, Napierala M, Hryciuk JE.** 2011. Modifications of commercial toxigenic *Clostridium difficile* PCR resulting in improved economy and workflow efficiency. J Clin Microbiol **49:**2279–2282. http://dx.doi.org/10.1128/JCM.00261-11.
13. **Qiagen.** 2014. QIAamp fast stool mini quick-start protocol DNA. Qiagen, Inc., Alameda, CA.
14. **Wenninger B, Munson E, Napierala M, Munson KL, Bilbo D, Hryciuk JE.** 2015. Frozen master mix-based modification of commercial reverse transcriptase PCR for detection of influenza and respiratory syncytial viruses. J Clin Microbiol **53:**1452–1453. http://dx.doi.org/10.1128/JCM.03457-14.
15. **Mahony J, Chong S, Jang D, Luinstra K, Faught M, Dalby D, Sellors J, Chernesky M.** 1998. Urine specimens from pregnant and nonpregnant women inhibitory to amplification of *Chlamydia trachomatis* nucleic acid by PCR, ligase chain reaction, and transcription-mediated amplification: identification of urinary substances associated with inhibition and removal of inhibitory activity. J Clin Microbiol **36:**3122–3126.
16. **Berg ES, GÅnestad Moi H, Størvold G, Skaug K.** 1997. False-negative results of a ligase chain reaction assay to detect *Chlamydia trachomatis* due to inhibitors in urine. Eur J Clin Microbiol Infect Dis **16:**727–731. http://dx.doi.org/10.1007/BF01709252.
17. **Monteiro L, Bonnemaison D, Vekris A, Petry KG, Bonnet J, Vidal R, Cabrita J, Mégraud F.** 1997. Complex polysaccharides as PCR inhibitors in feces: *Helicobacter pylori* model. J Clin Microbiol **35:**995–998.
18. **Munson E, Kramme T, Culver A, Hryciuk JE, Schell RF.** 2010. Cost-effective modification of a commercial PCR assay for detection of methicillin-resistant or -susceptible *Staphylococcus aureus* in positive blood cultures. J Clin Microbiol **48:**1408–1412. http://dx.doi.org/10.1128/JCM.02463-09.
19. **Munson E, Block T, Voegeli JT, Hryciuk JE, Schell RF.** 2009. Cost-effective frozen master mix modification of a commercial methicillin-resistant *Staphylococcus aureus* PCR assay. J Clin Microbiol **47:**1888–1891. http://dx.doi.org/10.1128/JCM.00506-09.
20. **Munson KL, Napierala M, Bilbo D, Munson E.** 2015. Frozen master mix modification of ProGastro SSCS assay for detection of *Salmonella* spp., *Shigella* spp., *Campylobacter* spp., and Shiga toxin-producing *Escherichia coli*. 115th General Meeting of the American Society for Microbiology. New Orleans, LA.