Viability Real-Time PCR as a Culture-Independent Diagnostic Tool in a Clinical Setting: A Pilot Study to Detect the Viability of *Salmonella* spp. in Stools

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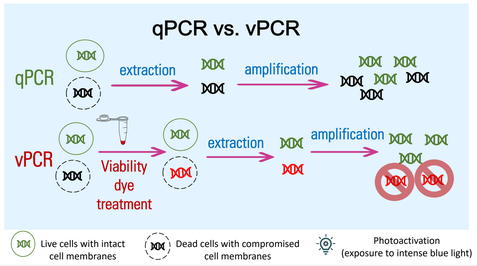
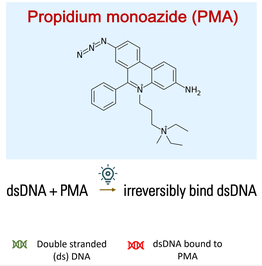
BACKGROUND & RATIONALE

About 4 million Canadians are infected with food-borne illnesses each year that lead to ~11,600 hospitalizations and ~240 deaths [1]. Accurate and timely diagnosis is crucial for prompt treatment and care. Culture-based diagnostic tests are time consuming and laborious and therefore, frontline microbiological diagnostic laboratories are moving towards culture-independent diagnostic testing (CIDT) for pathogen detection. Real-time PCR (qPCR) is commonly used in frontline labs due to high throughput, multi-analyte, automation, and a rapid turnaround time for reporting. However, the inability of qPCR to differentiate between DNA from live vs dead cells is a significant limitation as culture is required to confirm the pathogen viability.

A viability qPCR (vPCR) assay was developed to address this limitation by using a DNA intercalating dye, i.e. propidium monoazide (PMA) to remove dead cell DNA prior to DNA amplification by qPCR [2]. PMA is impermeant to intact cell membranes [2] and therefore, only live cell DNA will be amplified in a mixture of live and dead cells.

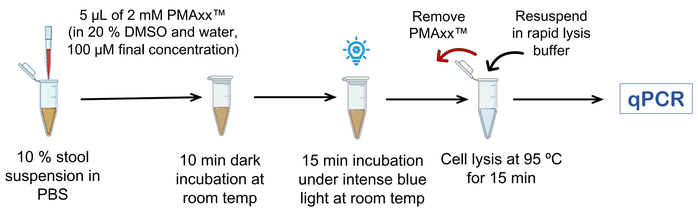
In our recent work, we optimized a vPCR assay to detect *Salmonella* Enteritidis in broth culture using PMAxx™as the viability dye [3]. Here, we sought to apply the vPCR protocol in a clinical setting to assess the viability of *Salmonella* spp. in diarrheal stools submitted by patients with acute gastroenteritis.



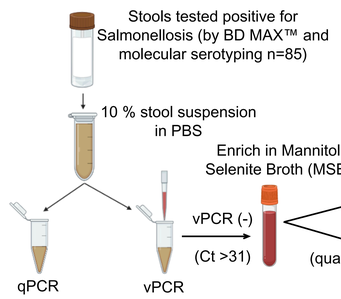


METHODS

Stool vPCR Protocol with PMAxx™ dye Treatment



Clinical Stool Analysis



Preparation of the Standard Curve

- *Salmonella* spp. negative stools (n=18)
- Stool environment: incubated in MSB at 37 ºC overnight.
- Spiked the incubated stool with different *S.* Enteritidis dilutions on the following day
- Analysis: qPCR analysis and culture (quadrant growth)

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RESULTS

The Sensitivity and Specificity of the In-House Primers and Probe

- Gene target [3]:
  - a conservative region from the *Salmonella invA* gene
  - No cross-reactivity
  - Limit of detection: 10^3 CFU/mL corresponding to a Ct cut off value of 31

The Standard Curve: qPCR and Quadrant Growth

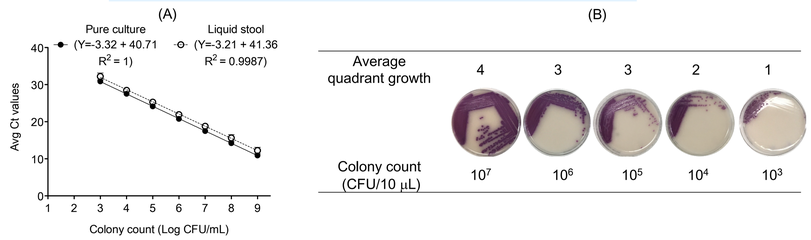


Figure 1. The qPCR standard curves and quadrant growth in CHROMagar™ Salmonella Plus plates. (A) Standard curve between average Ct values and average colony counts (CFU/mL) of *Salmonella enterica* subsp. *enterica* ser. Enteritidis in pure culture and liquid stools. n=18 with triplicates per qPCR run. Data is displayed as mean ± SD. Colony counts were confirmed by growth in culture. (B) The average quadrant growth on CHROMagar™ Salmonella Plus plates (n=18). Displayed growth on plates were not from the same replicate run. Scoring on plates were done in triplicate for each replicate. Ten microliters from each dilution was used for plating. Detection limit for culture: 10^3 CFU/10 µL. Ct: cycle threshold, CFU: colony-forming units.

Clinical Stool Analysis

Table 1: Summarized clinical stool data (n=85)

Group	Number of stools in group/Total no. analyzed
qPCR and vPCR positive	76/85
qPCR positive and vPCR negative	5/85
qPCR and vPCR negative	4/85

The Ct cut off for the qPCR limit of detection = 31; qPCR and vPCR positives: Ct <31; qPCR and vPCR negatives: Ct > 31; qPCR positive and vPCR negative: qPCR Ct <31 and vPCR Ct > 31.

Table 2. Pre- and post-enrichment data for vPCR negative stools (n=9)

Sample ID	Pre-enrichment data			Post-enrichment data	
	qPCR (Ct value)	vPCR (Ct value)	Stool culture	qPCR (Ct value)	Quadrant growth
3	34.48	36.34	POS	13.76	3
4	29.71	32.67	NEG#	16.20	3
9	30.53	33.08	POS	12.12	3
15	30.43	34.69	POS	12.16	3
22	31.36	33.73	POS	13.01	3
24	29.88	31.92	POS	12.15	4
29	27.39	30.70	POS	12.34	1
32	31.82	31.88	POS	12.53	4
33	UD	32.25	POS	13.20	3

Pre-enrichment qPCR and vPCR was done on the same day. Stool was cultured on CHROMagar™ Salmonella plus plates within 3 weeks of initial stool collection. #Culturing on plate was done > 30 days after initial stool collection. Post-enrichment qPCR and culture were done on the same day. Ct: cycle threshold; UD: undetected; POS: positive, NEG: negative

- Low bacterial loads in vPCR (-) stools (n=9):
  - confirmed by MSB enrichment overnight, subsequent qPCR analysis, and quadrant growth in CHROMagar™ Salmonella Plus plates.

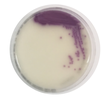
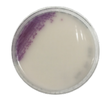
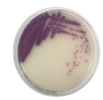
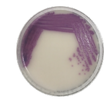
Sample ID	Quadrant growth	Sample ID	Quadrant growth	Sample ID	Quadrant growth
3		15		29	
4		22		32	
9		24		33	

Figure 2. Post-enrichment quadrant growth for stool samples with negative vPCR (n=9)

CONCLUSIONS & HIGHLIGHTS

- A viability PCR assay using PMAxx™ as the viability dye distinguished the presence of DNA from live vs dead *Salmonella* spp. in diarrheal stools.
- Presence of low loads of viable *Salmonella* spp. in stools as confirmed by MSB enrichment, led to higher vPCR Ct values.
- Stools were received in batches and therefore, *Salmonella* spp. in some stools could have lost viability by the time of analysis.
- With improved diagnostic sensitivity and further testing on other gastroenteric bacterial pathogens, vPCR has a great potential to become a multianalyte panel with the capability of assessing the viability of pathogens.

FUTURE DIRECTIONS

- Optimization of a stool-vPCR assay using *Salmonella* Enteritidis
- Testing the vPCR assay on other enteric pathogens such as Shiga-toxin producing *Escherichia coli*

References

[1] Thomas, M.K.; Murray, R.; Flockhart, L.; Pintar, K.; Fazil, A.; Nesbitt, A.; Marshall, B.; Tataryn, J.; Pollari, F. Estimates of Foodborne Illness-Related Hospitalizations and Deaths in Canada for 30 Specified Pathogens and Unspecified Agents. *Foodborne Pathog Dis* **2015**, *12*, 820–827.

[2] Nocker, A.; Cheung, C.Y.; Camper, A.K. Comparison of Propidium Monoazide with Ethidium Monoazide for Differentiation of Live vs. Dead Bacteria by Selective Removal of DNA from Dead Cells. *J Microbiol Methods* **2006**, *67*, 310–320.

[3] Thilakarathna, S.H.; Stokowski, T.; Chui, L. An Improved Real-Time Viability PCR Assay to Detect Salmonella in a Culture-Independent Era. *Int J Mol Sci* **2022**, *23*, 14708.

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