A Droplet Digital PCR (ddPCR) and Recombinase Polymerase Amplification (RPA) with Propidium Monoazide (PMAxx) for the Detection of Viable *Burkholderia cepacia* Complex in Nuclease Free Water and Antiseptics

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Abstract

Burkholderia cepacia complex (BCC) can survive and remain viable in both sterile and non-sterile pharmaceutical products. Subsequent presence of BCC has led to many outbreaks resulted in many product recalls. CDC has requested FDA to issue a rule or policy that establishes *Burkholderia cepacia* as an objectionable organism in pharmaceuticals, and United States Pharmacopeia (USP) has revisited the concept to include *B. cepacia* in its chapters. To reduce the frequency of such outbreaks, a quick and sensitive method for distinguishing between viable and non-viable BCC loads would be beneficial. Here, we used droplet digital PCR (ddPCR) and recombinase polymerase amplification exo (RPA exo) combined with propidium monoazide (PMAxx) for viable detection of BCC. The newly designed primers target Glycine betaine/L-proline transport system permease protein ProW for RPA exo and the ribB genes for ddPCR. 10 μM PMAxx and 5 min light exposure were sufficient in inhibiting signals from dead cells for ddPCR and RPA exo assay. The detection limit of ddPCR was as low as 9.7 fg/μL (97.2% specificity), while RPA exo at 40 °C for 20 min showed quantification of 10 pg/μL (80% specificity). PMAxx-ddPCR assay in nuclease-free water and antiseptics showed a sensitivity rage of 38 - 60%, compared to 74.6 - 85.0% without PMAxx ($p < 0.05$). PMAxx can be used as a nucleic acid dye to rule out the effects of amplifying dead bacteria by PMAxx-ddPCR and RPA exo assay. PMAxx-ddPCR can be used for detecting with a high degree of sensitivity, low numbers of cells, while RPA was quick, simple, and offers a new potential way of detecting BCC. The PMAxx-ddPCR and RPA exo assay developed in the present study, represents a new strategy to quantify live BCC cells directly in non-sterilized pharmaceutical products.

Introduction

- The *Burkholderia cepacia* complex (BCC) is a group of at least 24 closely related species characterized by a high metabolic versatility . They have emerged as one of the most reported contaminants of non-sterile pharmaceutical products posing a major health risk for many susceptible individuals . Several BCC outbreaks have been documented in recent decades, leading to the recall of multiple products . Furthermore, an examination of the U.S. Food and Drug Administration (FDA) Enforcement Reports (2012–2019) demonstrates that BCC is the primary source of non-sterile pharmaceutical product recalls. This led the FDA to propose the inclusion of these bacteria in the "Objectionable Microorganisms" category.
- Traditionally, BCC enumeration has been performed using culture-based procedures that are laborious and time-consuming and exhibit low sensitivity. Molecular techniques, such as PCR, quantitative PCR (qPCR), and droplet digital PCR (ddPCR), have shown great promise in BCC detection due to their pronounced sensitivity and specificity.
- Additionally, promising alternative to PCR, isothermal amplification of nucleic acids, achieves quick and effective amplification at a constant temperature, without the need for thermocycling.
- Nonetheless, a major drawback of PCR based amplification is its inability to distinguish between DNA originated from viable cells and DNA from dead cells . Dead bacteria do not cause serious diseases; rather, their presence leads to false positive results and inflated BCC counts . Therefore, we incorporated a sample treatment using propodium monoazide (PMAxx) to overcome this shortcoming. PMAxx is a DNA intercalating dye which penetrates membranes of damaged cells, forms covalent bonds upon exposure to bright visible light, and inhibits PCR amplification. In this study, we developed a droplet digital PCR (ddPCR) and recombinase polymerase amplification (RPA) with propidium monoazide (PMAxx) for the detection of Viable *Burkholderia cepacia* complex in nuclease free water and antiseptics.

Materials and Methods

Primer Selection

- For ddPCR, selected two primer set, RibB5 and RibB67 proved effective for amplifying the *ribB* genes
- For RPA, target Glycine betaine/L-proline transport system permease protein ProW

Specificity Analysis

- Evaluated the efficacy of the two primer sets, 20 BCC strains and 36 other bacterial strains (18 non-BCC, and 18 non-*Burkholderia*)

Optimization of PMAxx treatment

- Optimization of the PMAxx concentration and light exposure time which inhibit dead cells amplification: Concentrations of 10, 20, 30, 40, and 50 mM PMAxx; Light exposure times of 5, 10, and 15 min

Sensitivity/specificity Analysis

- Serial DNA dilution, ranging from 9.7 ng/μL to 9.7 fg/μL (9.7 ng/μL, 970 pg/μL, 97 pg/μL,9.7 pg/μL, 970 fg/μL, 97 fg/μL, 9.7 fg/μL) and amplified with the RibB67 primer set by PMAxx-ddPCR and RPA

Assessment of the PMAxx-ddPCR assay

- Assessment of the PMAxx-ddPCR Assay
  a. Using various ratios of Live vs. Dead *B. cenocepacia* J2315 cells
  b. Using different concentrations of CHX and BZK
- Application of the PMAxx ddPCR assay with 20 BCC strains in nuclease-free water and antiseptics

Results and Discussion

Optimization condition of ddPCR and RPA exo

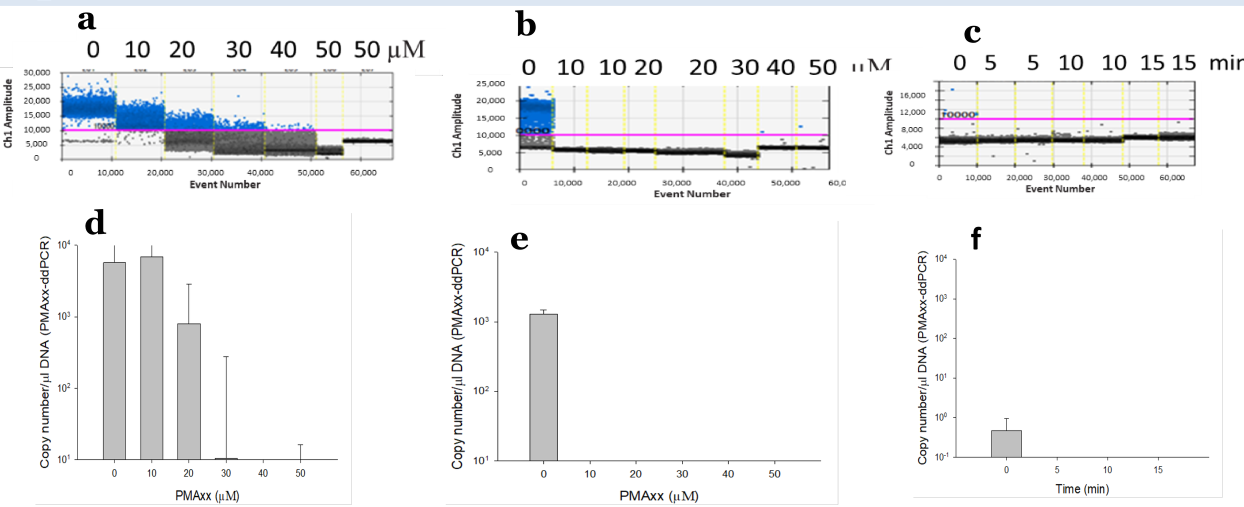

Figure 1 ddPCR. The effect of different concentrations of propidium monoazide (PMAxx) (**a**,**b**,**d**,**e**) and different LED light exposure times (**c**,**f**). The yellow vertical dotted lines separate results of individual reaction wells (**a**–**c**). DNA copy number obtained by ddPCR after exposing live (**a**,**d**) and dead (**b**,**e**) *B. cenocepacia* J2315 (1.5 × 10^8 CFU/mL) cells to different PMAxx concentrations (0, 10, 20, 30, 40, and 50 μM PMAxx). DNA copy number obtained by ddPCR after exposing dead *B. cenocepacia* J2315 (1.5 × 10^8 CFU/mL) to different LED light exposure times (0, 5, 10, and 20 min) (**e**,**f**).

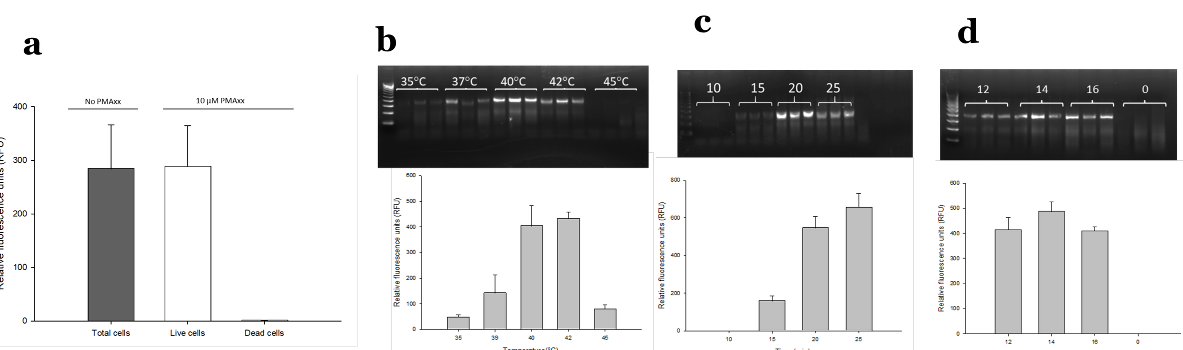

Figure 2.RPA (a) Assessment of 10 μM propidium monoazide (PMAxx) on live/dead B. cenocepacia J2315 (1.5 × 108 CFU/mL) cells in nuclease-free water. Total cells were left untreated (RPA exo assay) and live cells were treated with 10 μM PMAxx (PMAxx-RPA exo assay). (**b**)Optimization of temperature, (c) reaction time , and (d) concentration of magnesium acetate for the PMAxx-RPA exo assay using 0.9 ng/μL of B. cenocepacia J2315. RPA products were directly analyzed with the relative fluorescence units (RFU) by CFX96™ qPCR instrument. Results were confirmed by 2% agarose gel electrophoresis. A reaction was considered negative when the RFU value was below 50.

Limit of detection (LOD) of ddPCR and RPA exo

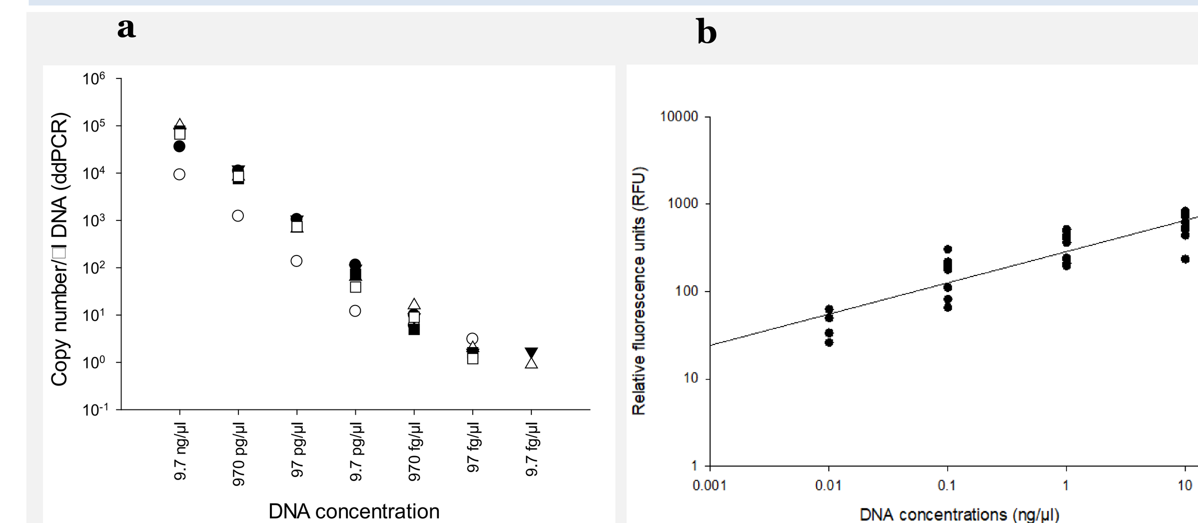

Figure 3. Limit of detection (LOD) of the PMAxx-ddPCR assay (**a**); and Limit of detection (LOD) of the PMAxx-RPA exo assay (**b**)

Specificity of ddPCR and RPA exo

Table 1. Specificity analysis using RibB67 primer (ddPCR) and gbpT (RPA) on 20 BCC, 18 Non-BCC, and 18 non-Burkholderia bacterial strains

Bacteria	No	Strain name	Source	Primer: ddPCR RibB67	Primer: RPA gbpT
BCC	1	*B. cepacia* PC783	Onion	+[a]	+
	2	*B. cepacia* AU24442	[b]CF sputum	+	+
	3	*B. stabilis* AU23340	CF sputum	+	+
	4	*B. pyrrocinnia* AU11057	CF sputum	+	+
	5	*B. ambifaria* HI2468	Pea, rhizosphere	+	+
	6	*B. anthina* HI2738	Soil rhizosphere	+	+
	7	*B. metallica* AU0553	CF sputum	+	+
	8	*B. metallica* AU16697	CF sputum	+	+
	9	*B. contaminans* HI3429	Sheep with mastitis, milk	+	+
	10	*B. contaminans* AU24637	CF lung	+	+
	11	*B. diffusa* AU1075	CF sputum	+	+
	12	*B. arboris* ES0263a	Soil	+	+
	13	*B. arboris* AU22095	CF sputum	+	+
	14	*B. lata* HI4002	Forest soil	+	+
	15	*B. cenocepacia* AU1054	CF blood	+	+
	16	*B. cenocepacia* AU0222	CF patient	+	+
	17	*B. cenocepacia* AU19236	CF sputu	+	+
	18	*B. cenocepacia* HI2976	Environment, sink	+	+
	19	*B. cenocepacia* HI2485	CDC sample	+	+
	20	*B. cenocepacia* J2315	CF Sputum	+	+
Non-BCC	21	*B. glumae* AU6208	CF lung nodule	-	+
	22	*B. plantarii* AU9801	CF Sputum	-	+
	23	*Caballeronia zhejiangensis* AU10475	CF Blood	-	-
	24	*Caballeronia zhejiangensis* AU12096	CF Blood	-	-
	25	*B. concitans* AU12121	CF Sputum	-	+
	26	*B. glumae* AU12450	Non-CF Lung	-	-
	27	*B. thailandensis* AU13555	Non CF infant Blood (wolfs parkinson white)	-	+
	28	*B. tropica* AU15822	CF Throat	-	-
	29	*B. gladioli* AU16341	CF Sputum	-	+
	30	*B. fungorum* AU18377	Non CF Jaw Aspirate	-	-
	31	*B. tropica* AU19944	CFSputum-Infant	-	-
	32	*B. gladioli* AU26454	CF Sputum	-	+
	33	*B. gladioli* AU29541	CF Sputum	-	+
	34	*B. gladioli* AU30473	CF Sputum	-	+
	35	*B. fungorum* AU35949	CF Endotracheal	-	-
	36	*B. thailandensis* AU36262	CF Sputum	-	+
	37	*B. plantarii* AU37486	CF Sputum	-	+
	38	*B. oklahomensis* ES0634	Environmental	+	-
Non-*Burkholderia*	39	*Enterococcus faecalis* ATCC29212		-	-
	40	*Enterococcus durans* ATCC6056		-	-
	41	*Proteus mirabilis* ATCC7002		-	-
	42	*Enterococcus faecium* ATCC35667		-	-
	43	*Bacillus subtilis* ATCC6051		-	-
	44	*Citrobacter frendii* ATCC8090		-	-
	45	*Pseudomonas aeroginosa* PAO1		-	-
	46	*Enterococcus faecium* ATCC49624		-	-
	47	*Yersinia enterocolitica subsp.entrocolitica* ATCC27729		-	-
	48	*Shigella sonnei* ATCC9290		-	-
	49	*Lactobacillus salivarius subsp. Salivarius* ATCC11741		-	-
	50	*Enterobacter aerogenes* ATCC13048		-	-
	51	*Klebsiella pneumoniae* ATCC13883		-	-
	52	*Pseudomonas aeroginosa* ATCC27853		-	-
	53	*Candida albicans* (Robin) Berkhout ATCC10231		-	-
	54	*Salmonella enterica*		-	-
	55	*Paenibacillus lautus*		-	-
	56	*Brevibacillus laterosporus*		-	-

Evaluated Live/Dead cells

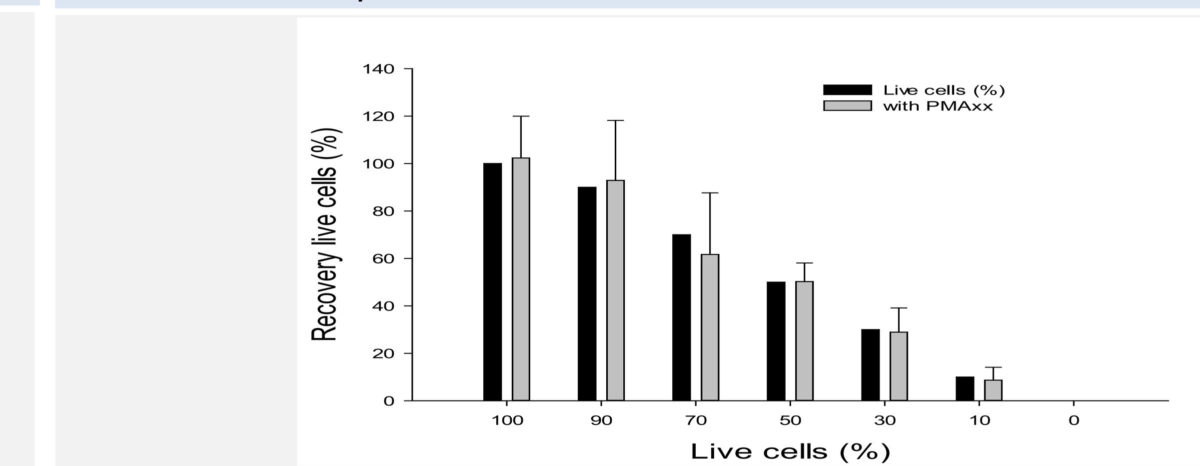

Figure 4. Effectiveness of PMAxx-ddPCR in selectively amplifying DNA from viable cells, mixtures of viable and dead cells were evaluated. Percentage of viable cells corresponded to 100, 90, 70, 50, 30, 10, 0%, after 10 μM PMAxx treatment.

Application of ddPCR in nuclease free water and antiseptics

Table 2. Estimated sensitivity analysis of the PMAxx- ddPCR assay from BCC cultured from nuclease-free distilled water, CHX, and BZK.

Tested Inoculum (CFU/mL)	Nuclease-Free Distilled Water		CHX		BZK	
	without PMAxx	with PMAxx	without PMAxx	with PMAxx	without PMAxx	with PMAxx
10^4	50/60 [a]	38/60	54/60	42/60	48/60	50/60
10^3	55/60	34/60	49/60	23/60	47/60	54/60
10^2	49/60	37/60	41/60	11/60	28/60	33/60
10	50/60	35/60	35/60	16/60	29/60	24/60
	204/240 (85.0%) [b]	144/240 (60.0%)	179/240 (74.6%)	92/240 (38.3%)	152/240 (63.3%)	161/240 (67.1%)
	$p = 0.0163$ [c]		$p = 0.0001$		$p = 0.7166$	

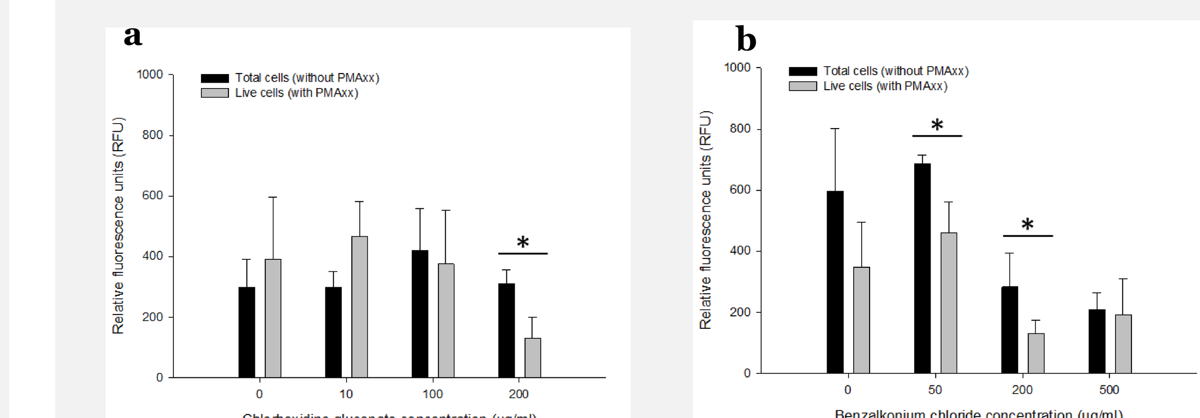

Figure 5. Comparison of cell lysates boiling DNA extraction method to a commercial Kit from different CHX concentrations. RFU from total cells (i.e., without PMAxx) (A), and live cells (i.e., with PMAxx) (B) treated with 5, 10, 50, 100 μg/mL CHX.

Conclusion

- The findings presented here, report two viability ddPCR and RPA assay for selective detection of live BCC in nuclease free water and antiseptics.
- 10 μM PMAxx and a 5-min LED light exposure were sufficient for screening viable BCC cells, particularly in nuclease-free water and 10 μg/mL CHX and 50 μg/mL BZK solutions.
- The PMAxx-ddPCR assay has 97.2% specificity and can quantify as few as 10 CFU/mL of BCC, which corresponds to an LOD of 9.7 fg/mL, while the PMAxx-RPA exo test was 80% specific and had a LOD of 10 pg equivalent to 10^4 CFU/ml.
- PMAxx-ddPCR can be used for detecting with a high degree of sensitivity, low numbers of cells, while RPA was quick, simple, and can be use as presumptive test for detecting BCC
- The developed assay could help determine the presence of BCC cells in pharmaceutical goods and provide a tool for risk-based health assessments in clinical settings.