MARSHALL UNIVERSITY

Evaluating DNA Preservation, Quantification and Inactivation of Methicillin-Resistant *Staphylococcus aureus* From a Collection and Transport System for Rapid Point-of-Care Diagnostic Tests

D. Brazeau [1,2], T. Stevens[2], T. Long [2], M. Karamchi[3],

[1] Medicine, Marshall University, [2]School of Pharmacy, Marshall University, [3]Puritan Medical Products, Guilford, ME

Introduction

Recent genomic technologies have made possible the accurate, rapid assessment of specific pathogens directly at the site of patient care. One of the major advantages of recent Point-of-Care (POC) instrumentation is their ease of use, making diagnostic tests accessible to personnel without specialized laboratory training or sample preparation. Gram-positive bacteria have been problematic for POC testing due to the difficulty of obtaining sufficient DNA for testing. The goal of this study was to assess the ability to detect methicillin-resistant *Staphylococcus aureus* (MRSA) DNA with minimal pretest preparation following collection and preservation in a MK buffered solution.

The goal of this project is

- To evaluate Puritan MK buffer solution's ability to preserve known concentrations of *Staphylococcus aureus* and allow recover of DNA with minimal pretest preparation.
- To assess the compatibility of DNA preserved in the media for both quantitative Polymerase Chain Reaction (QPCR) detection protocols and Loop-Mediated Isothermal Amplification (LAMP) detection protocols.
- To assess the rate of inactivation of *Staphylococcus aureus* (MRSA) folowing storage in Puritan MK buffer solution .

Methods

A methicillin-resistant *Staphylococcus aureus* (USA300 LAC JE2) suspension was prepared from fresh culture in a separate vial containing 10 mL of 0.85 % sterile saline and calibrated to a 0.5 McFarland Standard. 500 µl of eight ten-fold dilutions ranging from 1.5×10^7 to 1.5×10^3 CFU/ml were added to tubes containing 1 mL of MK buffered solution (3 replicates each) for storage for 0, 1, 7 and 30 days at two temperatures, 4° and 30° C. DNA was isolated using simple EtOH as the extraction method and quantified using Quantitative real-time PCR (Qiagen Microbial DNA qPCR Assay) and Loop-Mediated Isothermal Amplification (LAMP) protocols.

QPCR Protocol:

	one rxn		
QPCR Master mix (2x)	12.5	ul	
Primer Assay	1.0	ul	20.0 ul/sample + 5 ul *MRSA* DNA
Sterile H_2O	6.5	ul	
	20.0	ul	

Thermocycle Program

Enzyme activation	95 °C	10 min	
Denaturation	95 °C	15 sec	40 X
Annealing/Extension *	60 °C	2 min	* Detect FAM

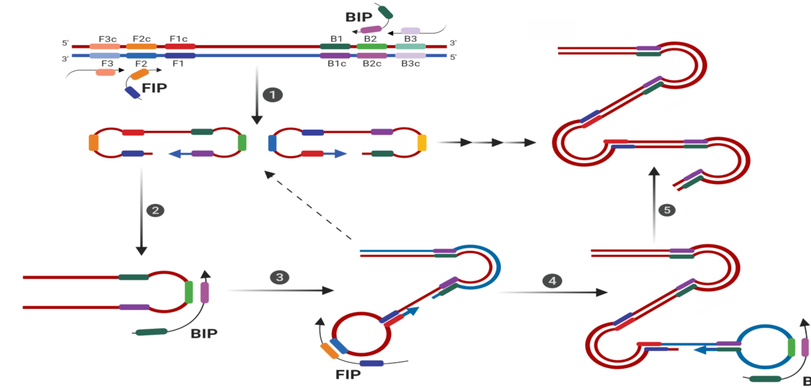
QPCR fluorescence readings were measured by BioRad CFX 96 Real-Time PCR System. The threshold for determining Ct value for each sample is based on the baseline threshold – above background and within the exponential phase of amplification curve.

Inactivation: MRSA samples at starting concentrations of 1.5×10^7 and 1.5×10^4 CFU/mL were used to assess the viability of MRSA stored in MK buffer. To determine the effect of concentration 1 ml of MRSA at each concentration was added to 1, 2, and 3 ml of MK buffer solution with three replicates each and incubated at RT for 1, 5, 10, 30, 60 and 180 minutes. At each time point 5 µl was then spotted onto growth plates and stored for 24 hrs at 37°C. Effective inactivation at each concentration and time point is defined as no detectable live cells.

Methods (continued)

Loop-mediated Isothermal Amplification (LAMP)

Six primer sequences for the detection of the *S. aureus* specific *mecA* gene were used (Misawa, Y. et.al. 2007). The LAMP assay was carried out in a 25 µl reaction mixture per manufacturers protocol using the WarmStart® LAMP Kit (New England BioLabs). 5 µl of the diluted samples were used for target detection using SYBR Green ® and an BioRad CFX 96Real-Time PCR System.



LAMP Protocol

MRSA Penicillin-binding protein 2'
From: Misawa, Y etal. 2007 J Infect. Chemother

mecA1 FIP: GGTCTTTCTGCATTCCTGGAATAATAGAAGATGGTATGTGGAAGT
mecA2 BIP: AGAACGTGGTAAAATTTTAGACCGACCTAATCTCATATGTGTTCCTGT
mecA3 F3: CATTGATCGCAACGTTCAA
mecA4 B3: AGATACATTCTTTGGAACGATG

For LAMP	one rxn	Lamp Cycle		
WarmStart Master mix (2x)	12.5ul			
Fluorescent dye - FAM (50x)	0.5ul	65 °C	10 sec	50 X
Lamp Primers (10x)	2.5ul	65 °C	1 min	* Detect FAM
Sterile H_2O	4.5ul			
	20.0ul	20 ul/sample + 5 ul cDNA or RNA		

Results

QPCR

All starting concentrations above 1.5×10^3 CFU/ml yielded quantifiable DNA from all replicates using either QPCR or LAMP protocols. At each concentration there was no significant difference in QPCR Ct values versus storage temperature (ANOVA, F=0.88, P=0.35). There was a significant difference in QPCR Ct values among the day 30 samples (ANOVA, F=30.88, P<0.001).

MRSA Limit of Detection

Final Concentration (CFU/mL)	Rep 1 Ct	Rep2 Ct	Rep 3 Ct	Rep 4 Ct	**Mean Ct**	**Std. Dev.**
7.5×10^7	25.2	25.8	24.9	24.1	**25.0**	**0.7**
7.5×10^6	26.6	27.1	28.7	29.4	**27.9**	**1.3**
7.5×10^5	30.8	32.6	31.6	31.0	**31.5**	**0.8**
7.5×10^4	37.5	37.3	34.6	35.8	**36.3**	**1.4**
7.5×10^3	39.2	38.5	39.3	37.8	**38.7**	**0.7**

Univariate Tests of Significance for Ct

Effect	SS	Degr. of	MS	F	p
Conc	1739.30	3	579.77	624.53	0.000000
Time	86.00	3	28.67	30.88	0.000000
Temp	0.82	1	0.82	0.88	0.352213
Conc*Time	24.94	9	2.77	2.98	0.005044
Conc*Temp	2.24	3	0.75	0.81	0.495473
Time*Temp	6.66	3	2.22	2.39	0.076846
Conc*Time*Temp	7.65	9	0.85	0.92	0.518029
Error	58.48	63	0.93		

MRSA Stability at 4° and 30° C

Final Concentration 1.5×10^4 CFU/mL

		Stability at 4°C			Stability at 30°C		
	Day 0	24 hrs	7 Days	30 Days	24 hrs	7 Days	30 Days
Mean Ct	36.4	36.6	35.8	39.6	35.7	35.9	38.5
Std Dev Ct	1.6	0.5	0.9	1.7	1.0	1.6	1.0

Final Concentration 1.5×10^5 CFU/mL

		Stability at 4°C			Stability at 30°C		
	Day 0	24 hrs	7 Days	30 Days	24 hrs	7 Days	30 Days
Mean Ct	31.7	32.7	32.5	34.0	33.0	32.5	34.5
Std Dev Ct	0.9	0.6	0.7	1.4	0.5	0.6	0.3

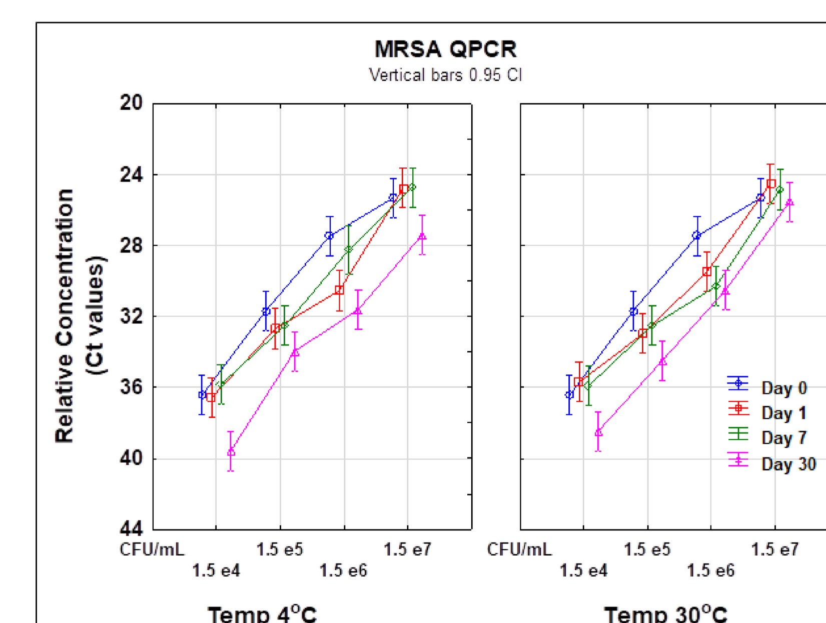
Final Concentration 1.5×10^6 CFU/mL

		Stability at 4°C			Stability at 30°C		
	Day 0	24 hrs	7 Days	30 Days	24 hrs	7 Days	30 Days
Mean Ct	27.5	30.3	28.3	31.6	29.5	30.3	30.5
Std Dev Ct	1.1	0.8	0.8	1.7	0.4	0.5	0.1

Final Concentration 1.5×10^7 CFU/mL

		Stability at 4°C			Stability at 30°C		
	Day 0	24 hrs	7 Days	30 Days	24 hrs	7 Days	30 Days
Mean Ct	25.3	24.8	24.7	27.4	24.5	24.9	25.5
Std Dev Ct	0.5	0.2	0.1	1.5	0.2	0.4	0.7

Plot of QPCR curves for MRSA at 4° and 30°C stored for 1, 7, and 30 days.



Results (continued)

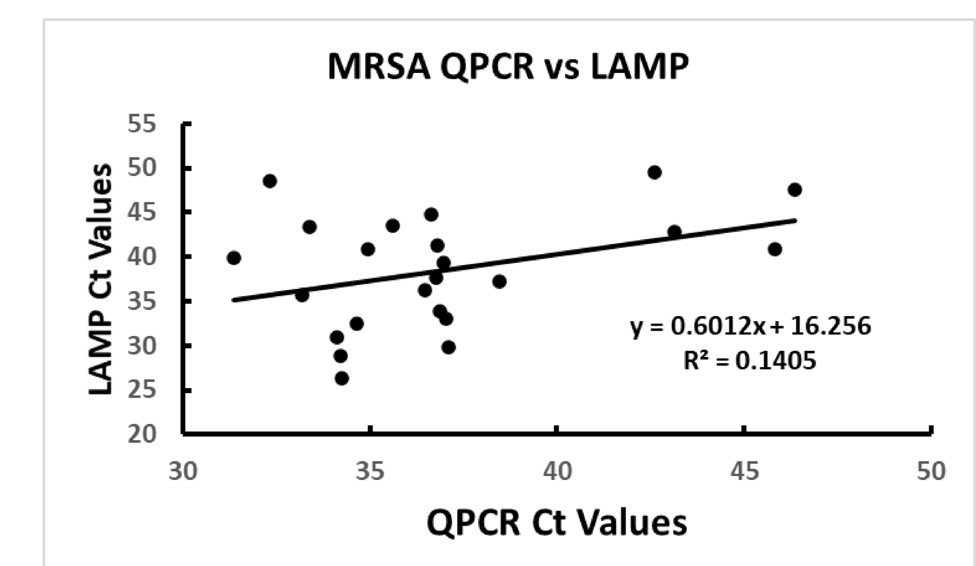
LAMP

Both QPCR and LAMP assays detected MRSA at all concentrations. However, while the QPCR was quantitative, the LAMP assay only indicated presence of the pathogen

Conc	N	Mean	Std Dev
1.5×10^7 CFU/mL	9	37.0	6.4
1.5×10^6 CFU/mL	8	40.3	6.6
1.5×10^5 CFU/mL	6	38.2	6.8
All	23	38.4	6.4

LAMP vs QPCR

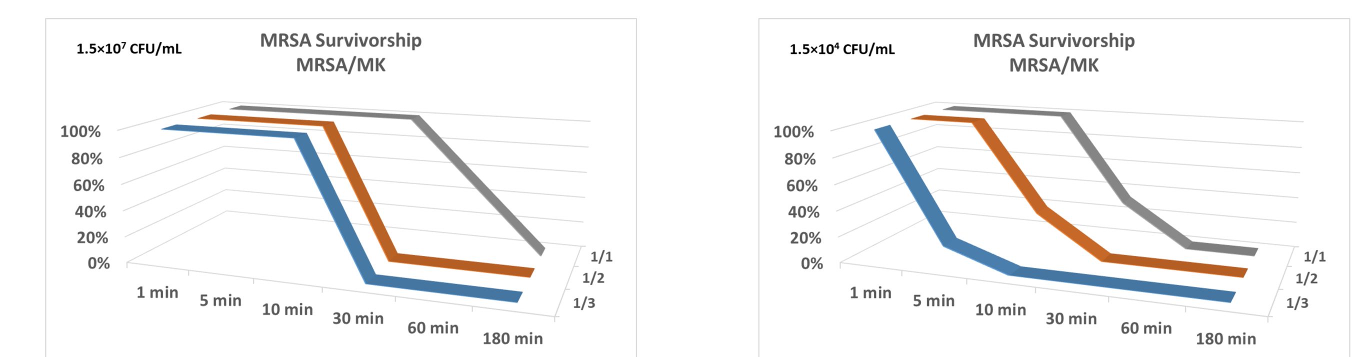
There was a nonsignificant postive correlation between QPCR and LAMP values ($r^2 = 0.14$).



Inactivation

Concentration Methicillin-Resistant *Staphylococcus aureus* (MRSA)	Dilution MRSA/MK Buffer	Exposure Time Growth after 24 hrs 3 relplicates (Positive / No Growth)					
		1 min	5 min	10 min	30 min	60 min	180 min
1.5×10^7 CFU/mL	1/3	3/3	6/6	3/3	0/6	0/6	0/3
	1/2	3/3	6/6	3/3	0/6	0/6	0/3
	1/1	3/3	6/6	3/3	6/6	3/6	0/3
1.5×10^4 CFU/mL	1/3	3/3	1/6	0/3	0/6	0/6	0/3
	1/2	3/3	6/6	1/3	0/6	0/6	0/3
	1/1	3/3	6/6	3/3	2/6	0/6	0/3

Complete inactivation of MRSA was observed after 180 minutes at either concentration with some growth among some samples at 30 and 60 minutes.



Conclusions

These results indicate that the MK buffer solution allows for the rapid detection of a Gram-positive bacterial pathogen of clinical relevance with minimal extraction procedures for use in QPCR and LAMP based POC testing following storage for up to 30 days at 30°C. Complete inactivation of *Staphylococcus aureus* (MRSA) occurred after storage for 3 hours at room temperature at concentrations ranging from 1.5×10^7 to 5.0×10^3 CFU/mL.

References

Misawa, Y. et at. (2007) Application of loop-mediated isothermal amplification technique to rapid and direct detection of methicillin-resistant *Staphylococcus aureus* (MRSA) in blood cultures. J. Infect ChemoTher 13:134-140

Acknowledgements

This project was supported by funding from Puritan Medical Products, Guilford ME.

printed by MegaPrint Inc. www.postersession.com