Development and Evaluation of Absolute qPCR for methicillin-resistant *Staphylococcus aureus* (MRSA) in Ventilator Associated Pneumonia (VAP) by using Mini-bronchoalveolar lavage (mini-BAL) Specimens

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Background

Methicillin-resistant *Staphylococcus aureus (*MRSA) is a common and critical pathogen responsible for many cases of ventilator associated pneumonia (VAP). VAP requires prompt treatment, and if initial antibiotics do not cover all isolated pathogens, mortality increases. The current available method "protected specimen quantitative cultures" require 72-96 hours for the full results. Therefore, empiric treatment with vancomycin for coverage of MRSA is often needed.

Objective

To facilitate rapid diagnosis while minimizing over utilization of antibiotics, we try to develop and evaluate an absolute qPCR assay for detection of MRSA in VAP using clinical Mini-BAL Specimens.

Materials and Methods

The protocol consists of:

1. Nucleic acid extraction from mini-BAL Specimens by using automatic system (NucliSens mini MAG, bioMérieux).
2. PCR Inhibitor detection in nucleic acid extracts by SPUD qPCR (Ref 1).
3. Development of MRSA absolute qPCR by using ABI 7900HT and SDS software v2.3.
4. Test the assay prospectively on 100 unused portion of mini-BAL samples taken during routine clinical practice.
5. Evaluate the results with protected specimen quantitative culture results.

Detection of Inhibitor in Nucleic Acid Extracted from Mini-BAL Specimens by SPUD qPCR

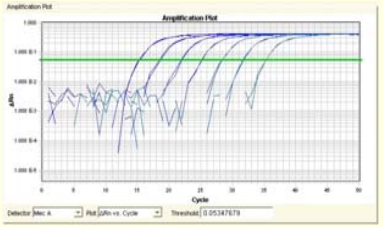
Among the many factors that determine the sensitivity, accuracy, and reliability of qPCR, template quality is one of the most important determinants of reproducibility and biological relevance. Inhibitory components frequently found in biological samples either will cause inaccurate quantitative results or create false-negative results. For solving this problem, a SPUD qPCR was used in this study to detect inhibitor in our nucleic acids extracted from mini-BAL samples.
SPUD qPCR is designed for the detection of inhibitors in nucleic acid extracted from all biological samples with exception of potato. The chosen amplicon sequences were located in the upstream nontranslated region of the S. tuberosum PhyB gene between nucleotides 449 and 549. The sequences of amplicon and primers are listed below. The location of primer and probe are indicated as underline.
SPUD Amplicon (101bp):
AACTTGGCTTTAATGGACCTCCAATTTTGAGTG*TGCACA AGCTATGGAACACCACGT*AAGACATAAAACGGCCACATAT GGTGCCATGTAAGGATGAATGT
Duplicate amplifications were carried out in 25ul which contains 1 pg SPUD synthesized amplicon, 500/100nM of SPUD primers/probe, 5ul isolated nucleic acid using QPCR master mix (Eurogentec) on ABI 7900HT sequences Detection Systems. The amplification without nucleic acid was used as a SPUD reference.

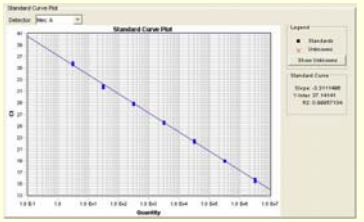
Principle of MRSA absolute QPCR

The MRSA absolute qPCR assay measures simultaneously three following targets*: mecA* gene, conferring methicillin resistance that is common to both *S. aureus* (*SA*) and *S. epidermis (SE) ; femA*–SA gene from *S. aureus*; *femA-SE* gene from *S. epidermis.* (Ref 2) reference controls and negative controls were included in each plate. Reference controls included MRSA and methicillin-resistant *SE (MRSE)*, and *mecA* gene standard curves obtained by serial dilution of plasmids containing known copy numbers of *mecA* gene fragments. The specificity of MRSA identification is based on the presence of the *mecA* gene and SA-specific *femA*–SA gene, with absence of SE specific *femA*-SE. This approach allows discrimination of the origin of the measured *mecA* signal and provides the copy number of *mecA gene.*

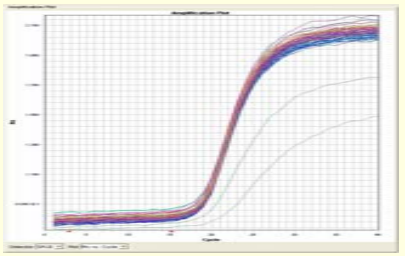
Results

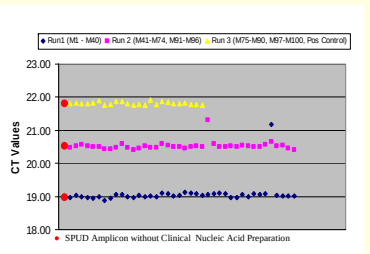
MecA Gene Amplification of Serially Diluted Plasmids inserted with *MecA* Fragment



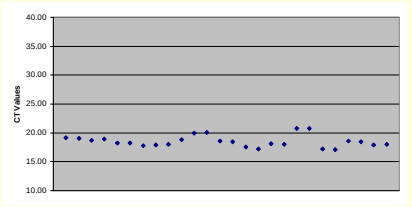


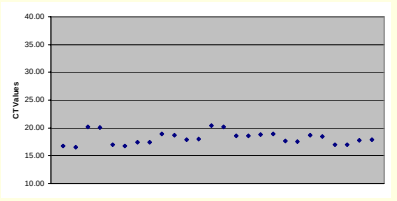
PCR Inhibitor Detection of Clinical Nucleic Acid Isolates by SPUD qPCR



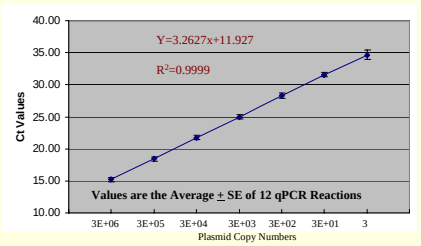


qPCR Results of *femA*-SA (left) and *femA*-SE (right) from MRSA Reference Strain (Clinical Isolate) In 12 Different MRSA Absolute qPCR Reactions

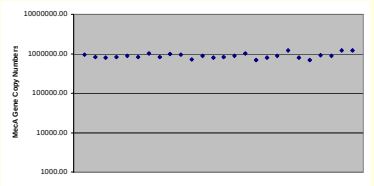




Linearity and Limits of Detection of MRSA Absolute qPCR



MecA Gene Copy Numbers of MRSE Reference Strain (Clinical Isolate) In 12 Different MRSA Absolute qPCR Reactions



Evaluate the MRSA Absolute qPCR Results with Protected Specimen Quantitative Culture Results

MRSA Absolute qPCR Interpretation of Clinical Samples

Sample ID	*femA*-SA Ct	*femA*-SE Ct	*mecA* Ct	*mecA* copy# /ml	interpretation
MRSE	UD	20.13+0.10	17.54+0.04	886890.90	MRSE
MRSA	19.96+0.08	UD	17.20+0.05	634055.70	MRSA
M1	UD	UD	UD	-	Neg
M2	31.05+0.12	UD	29.24+0.17	113.84	MRSA
M6	UD	34.23+0.29	35.85+0.75	1.37	MRSE
M9	UD	UD	37.34+0.78	0.78	Neg
M90	37.42+0.20	UD	UD	-	MSSA
M92	26.49+0.004	32.28+0.5	24.54+0.27	5206.26	MRSA+MRSE
M98	UD	37.89+0.23	UD	-	MSSE

Summary of MRSA Absolute qPCR

MRSA	MRSA+ MRSE	MRSE	MSSA	MSSE	Neg	Invalid	Total
19	5	8	1	3	62	2	100

MecA gene >0 copy/ml as cutoff

Overall Results of MRSA Absolute qPCR with Mini-BAL Specimens

Cutoff of MRSA Diagnosis	*mecA* gene copy number >0 as cutoff	*mecA* gene copy number >100 as cutoff
Sensitivity	90.48%	84.21%
Specificity	94.47%	98.70%
PPV	82.61%	94.12%
NPV	97.26%	96.20%

PPV: Positive predictive value NPV: Negative predictive value

Conclusion

PCR inhibitor in two nucleic acid extracts was detected By SPUD qPCR and excluded from this study.

Absolute qPCR for MRSA was able to distinguish MRSA from MRSE, MSSA, MSSE based on three genes amplification profiles and detect *mecA* gene down to a copy number of 3 with a high degree of linear correlation (R^2=0.999) in all 12 different runs.

Among valid 98 nucleic acid extracts from Mini-BAL specimens, there were 19 specimens with detectable MRSA, 5 specimens with MRSA and MRSE detection, 8 with MRSE, 1 with MSSA, 3 with MSSE and 62 samples were negative when we use *mecA* gene> 0 copy/ml as cutoff. Two samples were excluded from the study due to the presence of PCR inhibitor.

Using quantitative culture results as a "gold standard" for MRSA diagnosis, the sensitivity and specificity of absolute MRSA qPCR were 90.45% and 94.47% respectively. When we use >100 copies/ml *mecA* gene as cutoff for MRSA diagnosis in absolute qPCR, the positive predictive value increases from 82.61% to 94.12% without significantly sacrifice the negative predictive value (97.26% to 96.20%).

MRSA absolute qPCR is fast, highly sensitive, specific and quantitative assay for detection of MRSA in VAP by using mini-BAL specimens.

Reference

Ref 1 : Francois P, Pittet D, Bento M, Pepey B, Vaudaux P, Lew D and Schrenzel, J. Rapid Detection of Methicillin-Resistant *Staphylococcus aureus* Directly from Sterile or Nonsterile Clinical Samples by a New Molecular Assay. J Clin Microbiol. 2003 Jan; 41(1):254-260

Ref 2: Nolan T, Hands RE, Qgunkolade W and Bustin SA. SPUD: a quantitative PCR assay for the detection of inhibitors in nucleic acid preparations. Anal Biochem. 2006 Apr 15;351(2):308-10.

Support

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