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Polly Hockberger
Northern Michigan University, phockber@nmu.edu

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EVALUATION OF A MULTIPLEX REAL-TIME PCR ASSAY TO SIMULTANEOUSLY DETECT *STREPTOCOCCUS PNEUMONIAE* AND *STAPHYLOCOCCUS AUREUS* IN NASAL SAMPLES

by

Polly Hockberger

Thesis

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Abstract

Invasive disease caused by *Streptococcus pneumoniae* and *Staphylococcus aureus* is associated with nasal carriage of these bacteria. Use of a pneumococcal conjugate vaccine (PCV) in children altered nasal carriage rates and reduced infections due to *S. pneumonia.* Similar studies demonstrate that there is an inverse correlation between *S. pneumoniae* and *S. aureus* nasal carriage in children, but this inverse correlation has not been extensively investigated adults. The PCV is now approved for use in adults, a group more likely to be colonized with *S. aureus*. To investigate the impact of PCV vaccination on *S. aureus* carriage in adults, the clinical evaluation of a multiplex Real Time PCR method to simultaneously detect very low levels of *S. pneumoniae* and *S. aureus* from the same nasal sample is described.

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This thesis follows the format prescribed by the APA Style Manual (6th Ed.) and the Department of Clinical Laboratory Science.

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Introduction

Staphylococcus aureus is a leading causative agent of disease in humans, ranging from harmless skin infections to life-threatening systemic infections, and *S. aureus* is a commensal bacteria, colonizing about 30% of the human population (Tong, Davis, Eichenberger, Holland, & Fowler, 2015, Wertheim et al., 2005). Noskin et al. (2005) reports the burden of *S. aureus* on U. S. hospitals to be five times the risk of death, three times the length of stay, and three times the total cost: therefore, interventions that promote *S. aureus* colonization in humans may be increasing the burden of *S. aureus* infection. Some studies suggest a causal relationship between widespread use of pneumococcal conjugate vaccines (PCV7, PCV13, Prevnar®) and increased carriage rates of *S. aureus* due to the antagonistic relationship between *S. pneumoniae* and *S. aureus* (Creech, Kernodle, Alsentzer, Wilson, & Edwards, 2005, Spijkerman et al., 2012). This paper describes the evaluation of a novel multiplex real-time PCR (RT-PCR) assay designed to simultaneously detect *S. aureus* and *S. pneumoniae* from nasal samples to investigate the theory of in-vivo antagonism between the bacteria.

As reported by Marchisio, Esposito, and Shito (2002), *S. pneumoniae* is a commensal bacteria found in the nasopharynx of 73.1% of healthy children under two years old and 68.9% of healthy children aged 2-5 years. *S. pneumoniae* is also the leading cause of vaccine preventable death in the United States, with children under two years of age and adults over 65 being at highest risk for invasive pneumococcal disease (IPD) (Moore & Whitney, 2015). In 2001, the Advisory Committee on Immunization Practices for the CDC issued a new recommendation that all children aged 2-59 months be vaccinated against IPD using a conjugate pneumococcal vaccine. Prior to widespread

vaccinations, *S. pneumoniae* was responsible for 5,000,000 cases of acute otitis media, 17,000 cases of invasive disease, and 200 deaths annually (Centers for Disease Control and Prevention, 2015). While overall *S. pneumoniae* carriage rates have remained stable, decreases in the invasive serotypes covered by the vaccine have decreased the incidence of IPD in children under five years from 100 cases per 100,000 in 1998 to 9 cases per 100,000 in 2015 (CDC, 2015).

Long known as the cause of human infections, the epidemiology of *S. aureus* infections shifted in the 1990's to emphasize healthcare associated infections, especially those associated with infective endocarditis, prosthetic device infections and community associated skin and soft tissue infections, and the relationship between commensally carried *S. aureus* including methicillin resistant *S. aureus* (MRSA) and its connection to disease. (Tong et al., 2015, Wertheim et al., 2005). Genotyping studies of *S. aureus* suggest a causal relationship between nasal carriage of *S. aureus* and infection (Wertheim et al., 2005). MRSA was initially isolated in the early 1960's shortly after the introduction of methicillin, and is particularly concerning to public health agencies due to its resistance to beta lactam inhibitor antibiotics (David & Daum, 2010). MRSA is now recognized in two forms: healthcare-associated MRSA (HA-MRSA) defined by the CDC (2015) as MRSA isolated within four calendar days of admission to a healthcare facility, or two days after central line placement, and community associated MRSA (CA-MRSA) defined by the CDC (2015) as MRSA infection isolated from patients without healthcare facility risk factors. The CDC reports that two percent of the U.S. population are nasal carriers of MRSA. According to the emerging pathogen report published by the CDC in 2014, MRSA caused 72,444 cases of infectious disease in the U.S. with an annual

incidence rate of 22.72 in 100,000. The overall incidence of MRSA has decreased by over 39% since 2005 driven in large part to health care facilities' infection control practices designed to prevent the spread of MRSA (CDC, 2015; David & Daum, 2010). The number of CA-MRSA cases continues to increase (CDC, 2015).

While the anterior nares serve as reservoirs for both *S. aureus* and *S. pneumoniae*, the two organisms have an antagonistic relationship (Selva et al., 2009). An in vitro study by Selva et al. (2009), suggested that H_2O_2 production by *S. pneumoniae* was responsible for the lysogenic response by *S. aureus*. However, Reiss-Mandel and Regev-Yochay (2015) suggested that in vivo studies support a host immune mediated response as a probable explanation of the seeming competitive nature between the two organisms. Of particular note was the inverse correlation the authors reported between *S. aureus* and *S. pneumoniae* strains that have a pilus, as piliated strains tend to be more virulent. Many of these virulent, piliated *S. pneumoniae* strains are also included in the pneumococcal vaccine (Reiss-Mandel & Regev-Yochay, 2015).

From 2002 to 2007, within two years of routine PCV administration to children, Gerber, Coffin, Smathers, and Zaoutis (2009) reported a 300% increase in MRSA infection rates in hospitalized children. It is possible that PCV contributed to the increase in MRSA infections due to a shift in balance between these competitive niche bacteria. Nasal carriage of either *S. pneumoniae* or *S. aureus* has been linked to invasive disease from the carried organism (Kluytmans, van Belkum, & Verbrugh, 1997). Spijkerman et al. (2012) reported a higher incidence of *S. aureus* in nasopharyngeal swabs from children aged 11 months and 24 months who received the pneumococcal conjugate

vaccine, and their parents when compared to samples collected prior to vaccine administration.

In 2011, the CDC expanded the use of conjugate pneumococcal vaccine to include all persons over the age of 50 to further reduce the incidence of IPD. At the time of this study, there were no studies comparing the pre- and post-vaccination carriage rates of *S. aureus* and *S. pneumoniae* among adults to the author's knowledge.

Previous studies comparing *S. pneumoniae* and *S. aureus* carriage rates were performed using culture methods. Unlike using a culture to detect overt infection, cultures to detect carrier status may be unreliable due to growth of complex commensal respiratory flora. *S. pneumoniae* can be especially difficult to distinguish from other normal nasopharyngeal flora, such as *S. viridans* when using traditional culture methods (CDC, 2015). Recent studies support RT-PCR methods as having superior sensitivity than culture methods. No commercially available multiplex RT-PCR test to simultaneously detect *S. pneumoniae* and *S. aureus* from nasal samples was available at the time of this study. Here the evaluation of a multiplex RT-PCR assay previously designed in the Northern Michigan University Laboratory to detect low levels of *S. aureus* and *S. pneumoniae* carriage in clinical nasal samples is described (Pierpont, 2016).

Methods

This study was approved by the Institutional Review Board of Northern Michigan University to include consenting adults (Appendix A). Volunteer test subjects were given a questionnaire (Appendix B) to establish age, gender and race. Additional questions were asked to note previous infection due to *S. aureus*, or *S. pneumoniae*, or co-

morbidities such as cancer, bone marrow or organ transplantation, current infections requiring antibiotic treatment, and finally PCV immunization status.

Repeat sampling from multiple sites is recommended in order to identify transient carriers of *S. aureus* (Wertheim et al., 2005). All volunteer subjects used rayon tipped swabs (Copan) to self-collect one sample from each nostril by gently inserting a swab into the exterior nares, sweeping upwards and rotating around the circumference of the nares prior to removal.

All swabs were directly plated on 5% sheep's blood agar (SBA) media (Remel). Plates were incubated at 35 to 37°C. Plates were inspected at approximately 24 and 48 hours for alpha hemolytic colonies suspicious for *S. pneumoniae* and beta hemolytic colonies suspicious for *S. aureus.* Subcultures of suspicious colonies were plated on 5% SBA to ensure culture purity for serological testing. The Oxoid Dryspot Pneumo Kit™ was used to positively identify *S. pneumoniae*. The Remel Staphaurex® test kit was used to positively identify colonies suspicious for *S. aureus*. Isolates that tested positive by Staphaurex® were plated to mannitol salt agar (MSA) for confirmation by growth and fermenter status.

After primary plating for culture, swabs were placed in Tryptic Soy Broth (TSB) as an enrichment media and incubated overnight at 37°C. Aliquots of the inoculated TSB media were frozen pending DNA extraction. Following the QIAamp® DNA Mini Kit (Qiagen) instructions, 100 ml of inoculated TSB was used for bacterial DNA extraction. Extracted DNA was immediately frozen at -70°C pending RT-PCR analysis. A portion of TSB from volunteer samples were reserved for subculture to MSA plates to correlate the quantitation cycle (Cq) from *nuc* positive samples to a culture detectable limit.

Additionally, due to the low recovery of *S. pneumoniae* positive clinical samples by culture method, nasal samples that were negative for *S. aureus* and *S.pneumoniae* by both culture and multiplex RT-PCR were spiked with *S. pneumoniae* in concentrations ranging from 1.53 x 10^6 copies per microliter through 1.53 x 10^2 copies per microliter. A nasal sample that was positive for *S. aureus* by culture and *nuc* positive by multiplex RT-PCR was spiked with the same dilutions of *S. pneumoniae.*

Genes selected for target analysis were based on PubMed searches of current literature. The thermonuclease (*nuc*) gene was selected as a recommended target region to identify *S. aureus* (Chesneau, Allignet, & El Solh, 1993). A *nuc* sense 5'-GTTGCT-TAGTGTTAACTTTAGTTGTA-3'and antisense 3'- TTTAATGTATTTCTTGGA-CGCTGTAA -5' primer set previously characterized by Kilic, Muldrew, Tang, and Basustaoglu (2009) was used in the multiplex RT-PCR analysis. Repeated testing with known *S. aureus* strains produced amplicons with melting peaks between 78.28°C and 78.96°C. The CDC recommends the *lytA* gene to identify *S. pneumoniae*. Biosoft Beacon Designer software was used to design a primer set, *lytA* sense 5'- CACGAATAACCA-ACCAAA -3' and antisense 3' –TAGCCAGTGTCATTCTTC- 5', to detect the *lytA* gene with an amplicon melting peak range of 81.90°C to 82.62°C that showed no cross reactivity with the *nuc* primer set. All primer sequences were analyzed with NCBI Blast program to rule out potential cross reactivity with human DNA. The sequences were manufactured by IDT (Table 1).

IDTE buffer was added to equal parts of the *lytA* and *nuc* primers to achieve a 5.0 micro-molar concentration. Each reaction used 3µL molecular grade water, 2µL of the primers for a final concentration of 0.5 µM, and 10µL of Green DNA Master. The Roche

LightCycler 96 was used with FastStart Essential Green DNA Master (Roche Applied Science). RT-PCR cycling conditions were as follows: initial denaturation at 95°C for 600 seconds with a ramp rate of 4.4°C per second, followed by 45 cycles of a three step amplification including 95°C for 10 seconds with a ramp rate of 4.4°C per second, 62°C for 10 seconds with a ramp rate of 2.2°C per second, and 72°C for 10 seconds with a ramp rate of 4.4°C per second, followed by a final melting curve comprised of 95°C for 10 seconds with a ramp rate of 4.4°C per second, 65°C for 60 seconds with a ramp rate of 2.2°C per second, and 97°C for one second with a ramp rate of 0.1°C per second (Table 2).

Results

A multiplex RT-PCR assay to detect low levels of *S. aureus* and *S. pneumoniae* was previously developed in our laboratory (Pierpont, 2016). The sensitivity of the assay was established by testing serial dilutions of six *S. aureus* controls and three replicates of two *S. pneumonia* controls with known DNA concentrations. Initial evaluation data suggests the assay functions as designed and that it can reliably detect 10 copies of *S. aureus* DNA and 100 copies of *S. pneumoniae* (Figure 1, Figure 2). In order to assess the performance of the assay in detecting *S. aureus* and *S. pneumoniae* in samples that more closely mimic clinical conditions, the results of the multiplex real-time assay were compared with those obtained by conventional culture and phenotypic identification methods using nasal swabs self-collected from 147 adult volunteers at a college campus. *S. aureus* carriage was detected by culture in 41 of 218 samples (22.4 %) and of these 38 tested positive by multiplex PCR yielding a clinical sensitivity of 92.7% for the PCR based method when compared to culture (Table 3). The acceptable *nuc* amplicon melting

range was expanded to 77.80°C through 79.20°C based on the melting points of samples that were positive for *S. aureus* by both culture and PCR (Figure 3). The results of the remaining volunteer samples were considered positive for *S. aureus* by PCR if detected by quantification cycle (Cq) 35.00 and the amplicon's melting point was within the revised range. All volunteer samples were evaluated for *S. aureus* using these criteria. *S. aureus* was detected by multiplex RT-PCR in 177 of the 218 samples (Table 4). When the carriage rate is assessed by both culture and RT-PCR, 33 of 147 volunteers were *S. aureus* positive by culture yielding a carriage rate of 22.4 %, whereas the number of volunteers with a positive sample as determined by RT-PCR was 139, a 94.6% carriage rate (Table 4). Although studies have shown that PCR methods are more sensitive at detecting the presence of low bacterial loads than culture methods, the number of samples positive for *S. aureus* was significantly higher than the currently accepted *S. aureus* carriage rate of 30-50% based on culture so additional testing was warranted to verify the findings. (CDC, 2015; Kumar et al., 2008).

To confirm the unusually high carriage rate of *S. aureus,* we selected 50 samples that were positive by multiplex RT-PCR at a range of Cq values were re-tested by uniplex PCR using three separate primer sets. The PCR reactions used a unique *nuc* primer set developed with Biosoft Beacon Designer software, a *nuc* primer set previously characterized by Hoegh et al. (2014), and the *nuc* primer set previously characterized by Kilic et al. (2009), that was used in the multiplex RT-PCR assay. BLAST analysis confirmed that each primer set assayed for separate regions of the *S. aureus nuc* gene and the amplicons did not contain overlapping sequences. Samples were considered positive for *S. aureus* if the Cq value was less than 35.00 and the amplicon melt peak

corresponded to that generated by positive controls (Figure 2). The uniplex Kilic primer set detected *S. aureus* in 48 of 50 samples, the Hoegh primer set detected *S. aureus* in 47 of the 50 samples, and the Biosoft primer set detected *S. aureus* in 39 of the 50 samples. *S. aureus* was detected in 39 of the 50 samples with all three uniplex primer sets (Table 5). Amplification was detected at similar Cq values for the majority of samples assayed by all three primer sets. Data gathered from the previous evaluation studies suggest the Biosoft *nuc* primers were less efficient, and subsequently less sensitive for *S. aureus* than the Kilic or Hoegh *nuc* primer sets (data not shown). These findings conservatively suggest that at a minimum the *S. aureus nuc* gene was present in 75% of the volunteer samples with positive calls using the multiplex RT-PCR assay.

The *nuc* gene is highly conserved in most *S. aureus* strains and many studies have verified its utility in the detection of *S. aureus* (Kilic et al., 2009; Menzies, 1977). To verify that other bacteria commonly carried in the nose were not causing false positive results, TSB aliquots from 13 multiplex RT-PCR *nuc* positive volunteer samples were sub-cultured to MSA plates and cultured overnight. *S. aureus* growth was observed in MSA sub-culture from the three volunteer samples, six samples grew non-aureus *Staphylococcus*, and four exhibited no growth after overnight incubation (Table 6). DNA extracted from the non-*S. aureus* culture growth isolated on MSA were all negative for the *nuc* gene with the multiplex RT-PCR assay, suggesting that the multiplex RT-PCR positive results were due to the presence of *S. aureus.* Collectively these experimental results suggest that as many as 76% of the study volunteers may be carriers of *S. aureus* (Table 6).

There was insufficient data to evaluate the sensitivity of the multiplex RT-PCR assay to detect *S. pneumoniae* as only five nasal samples obtained from the volunteers were positive by culture. Only three of the five culture positive samples were available for PCR analysis and of the three, *S. pneumoniae* was detected in only two. An additional seven samples were positive for *S. pneumoniae* by multiplex RT-PCR that were culture negative (Table 7). To further evaluate the detection sensitivity for *S. pneumoniae* with the multiplex RT-PCR assay, two volunteer nasal samples, one that was negative for *S. pneumoniae* and *S. aureus* by both PCR and culture, and one that was negative for *S. pneumoniae* and positive for *S. aureus* by culture and PCR, were spiked with *S. pneumoniae* at concentration ranging from 1.53×10^6 copies per microliter through 1.53×10^2 copies per microliter. *S. pneumoniae* was detected in the spiked nasal samples negative for *S. aureus* at 1.53×10^6 copies per microliter through 1.53×10^3 copies per microliter (Table 8). *S. pneumoniae* was not detected at 1.53×10^2 copies per microliter. No *S. pneumoniae* was detected in the in any of the *S. aureus* positive nasal samples that were spiked with *S. pneumoniae*.

Discussion

This study was performed to establish the usefulness of a multiplex RT-PCR assay to simultaneously detect very low levels of *S. aureus* and *S. pneumoniae* from enriched nasal cultures by comparing the detection of these bacteria by conventional culture and RT-PCR assay. The assay is relatively sensitive for detection of *S. aureus;* of 41 samples positive for *S. aureus* by culture, 38 were positive by multiplex RT-PCR yielding a clinical sensitivity of 92.7 percent, a value slightly below optimal, but within

acceptable limits per Clinical Laboratory Standards Institute guidelines (2008). Notably, *S. aureus* was detected by multiplex RT-PCR in 139 out of 177 culture negative samples. Multiple studies have demonstrated that nucleic acid amplification based methods are superior to culture for detection of low level bacterial carriage, especially in asymptomatic individuals (Kumar et al., 2008). The unexpectedly high rate of detection prompted the performance of follow up experiments to determine the presence of the thermonuclease gene and to rule out the presence of a homologous thermonuclease gene present in another organism in the nasal microbiome. Detection of *S. aureus* at comparable Cq values by all three uniplex PCR assays suggest that the thermonuclease gene was present in at least 75% of the samples. Multiplex RT-PCR results were all negative from the extracted DNA of non-*S. aureus* organisms as identified by MSA sub-culture suggest that the thermonuclease gene detected is specific to *S. aureus.* The Cq values of RT-PCR positive samples were compared to *S. aureus* detection by culture. In 28 of 41 culture positive samples, initial multiplex RT-PCR analysis detected *S. aureus* at a Cq $\leq$ 20.00. As the level of detection for *S. aureus* control samples was determined to be as low as 10 DNA copies by Cq 35.00, samples that tested positive by Cq 35.00 can be considered true positives with a reasonable level of assurance. Of the culture positive samples, 28 were positive for *S. aureus* by PCR Cq at or before Cq 20.00. This finding suggests that a significantly higher bacterial burden is required for detection by culture than is needed for detection by RT-PCR. It also suggests that *S. aureus* was present at levels below culture limits of detection in volunteer samples that tested positive by multiplex RT-PCR at a Cq greater than 20.00 and below 35.00.

The multiplex RT-PCR assay did not efficiently amplify the *lytA* gene found in *S. pneumoniae*. Samples were culture positive for *S. pneumoniae* from three volunteers, whereas the multiplex RT-PCR assay was positive for *S. pneumoniae* in only two of the three volunteers. *S. pneumoniae* was detected in seven volunteer samples that did not have culture positive results, however, all of the Cq values for *lytA* amplification were greater than 20.00 and the inability of the assay to detect *S. pneumoniae* in samples positive for *S. aureus* suggest an inefficient *lytA* primer set.

S. aureus was detected in at least 76% of the volunteers using the novel multiplex RT-PCR assay. Contributing factors to such a high rate of detection include sample collection from multiple sites to increase the probability of detecting transient carriers. This hypothesis is supported by the finding that collecting swabs from both nostrils of the study volunteers increased the likelihood that both persistent and transient carriers of *S. aureus* or *S. pneumoniae* were identified (Senn, Bassett, Nahimana, Zanetti, & Blanc, 2012). As noted by Kumar et al. (2008) the use of enrichment media also increases sensitivity and overall bacterial detection rates in PCR analysis.

Use of separate swabs to inoculate primary culture plates and TSB enrichment media is recommended and the use of a single swab for both may account for the multiplex RT-PCR assay having lower than expected sensitivity rates for both *S. aureus* and *S. pneumoniae* in culture versus multiplex RT-PCR (Sherlock, Dolan, & Humphreys, 2010). An additional limitation that may have contributed discrepant results was the use of non-flocked rayon swabs were used to collect study samples. It has been suggested that a nylon flocked swab is a more appropriate collection device when employing PCR

to detect *S. pneumoniae* because the shorter fibers release organism more readily than the longer fibers comprising non-flocked swabs (Dube, Kaba, Whittaker, Zar, & Nicol, 2013). Finally, a manual method for DNA extraction was used that may be more vulnerable to pre-analytical DNA contamination and as noted by van Tongeren, Degener, and Harmsen (2011) the efficiency of the QIAamp® DNA Mini Kit (Qiagen) manual DNA extraction method used with the nasal samples can be as low as 7.7% compared to other extraction methods.

Conclusions

Results of the study suggest that the multiplex RT-PCR assay for the detection of *S. aureus* and *S. pneumoniae* is more sensitive than culture. Despite the higher sensitivity of the multiplex RT-PCR assay, results of the study do not support the use of this multiplex RT-PCR assay as a screening method for *S. aureus* and *S. pneumoniae* carriage due to the limited efficiency of the *S. pneumoniae* primer set in detecting very low levels of *S. pneumoniae*, especially in mixed cultures that contain *S. aureus*.

Evidence of *S. aureus* carriage rates based on RT-PCR analysis appear significantly higher than rates previously reported based on culture. To our knowledge, published data states that only 30% of the population are permanent carriers of *S. aureus* and an additional 20 to 30% of the population are transient carriers. *S. aureus* carriage in at least 75% of study volunteers on a university campus will be explored more fully in future experiments using DNA extracted from frozen enrichment broth aliquots of the volunteer samples performed using an efficient, automated method. If repeat analysis with the *nuc* primer sets yield results similar to the results previously obtained, the

presence of *S. aureus* will be confirmed with a Taqman based probe assay or sequencing and a novel LAMP assay to detect the *nuc* gene and possibly additional gene targets specific to *S. aureus.* An investigation of the clinical significance of *S. aureus* carriage density below levels detectable by culture and possible genetic or environmental factors that predispose some persons to *S. aureus* infection associated with commensal carriage may be indicated.

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Table 1: Primer sequences

Primers in Multiplex	***Sequence***
Kilic *nuc* forward primer	5’-GTTGCTTAGTGTTAACTTTAGTTGTA-3’
Kilic *nuc* reverse primer	3’- TTTAATGTATTTCTTGGACGCTGTAA -5’
Biosoft *lytA* forward primer	5’- CACGAATAACCAACCAAA -3’
Biosoft *lytA* reverse primer	3’- TAGCCAGTGTCATTCTTC - 5’
Primers in Uniplex	***Sequence***
Hoegh *nuc* forward primer	5’ - GGTTCTGAAGATCCAACA - 3’
Hoegh *nuc* reverse primer	3’ - GTCTGAATGTCATTGGTTG -5’
Biosoft *nuc* forward primer	5′- GGGTTGATACGCCAGAAACG -3′
Biosoft *nuc* reverse primer	5′-TGATGCTTCTTTGCCAAATGG -3′

Table 2: Roche LightCycler 96 settings

LightCycler Settings			
Setup			
Run Editor			
Detection Format			
Dyes 1:	SYBR Green		
Profile			
Programs			
Temp (°C)	Ramp (°C/s)	Duration (s)	Acquisition Mode
Pre-incubation			
95	4.4	600	None
3-Step amplification			
No. of Cycles: 45			
95	4.4	10	None
62	2.2	10	None
72	4.4	10	Single
Melting			
95	4.4	10	None
65	2.2	60	None
97	0.1	1	Continuous

Table 3: Assay clinical sensitivity for *S. aureus* detection

Samples	RT-PCR positive (n=177)	RT-PCR negative (n=41)
Culture positive (n=41)	38	3
Culture negative (n=177)	139	38

Table 4: *S. aureus* carriage rates by method

Volunteers	Culture positive	Multiplex RT-PCR positive
147	33	139
% Carriage	22.4%	94.6%

Table 5: Uniplex assay result comparison

Total Samples	Multiplex (Kilic)	Kilic – uniplex	Hoegh – uniplex	Biosoft - uniplex
50	50	48	47	39

Table 6: MSA sub-culture results

No growth	Non-fermenter (non-*aureus Staphylococcus*) present	Fermenter (*S. aureus pos)*
4	6	3
nuc gene positive	0	3 (initial multiplex result)

Table 7: *S. pneumoniae* results

Samples	RT-PCR positive (n=9)	RT-PCR negative (n=209)
Culture positive (n=3)	2	1
Culture negative (n=215)	7	208

Table 8: Results of nasal samples spiked with *S. pneumoniae*

S. pneumoniae concentration	Tm °C	Cq
1.53×10^6	82.34	21.71
1.53×10^5	82.38	28.31
1.53×10^4	82.41	30.96
1.53×10^3	82.43	34.66
1.53×10^2	-	-

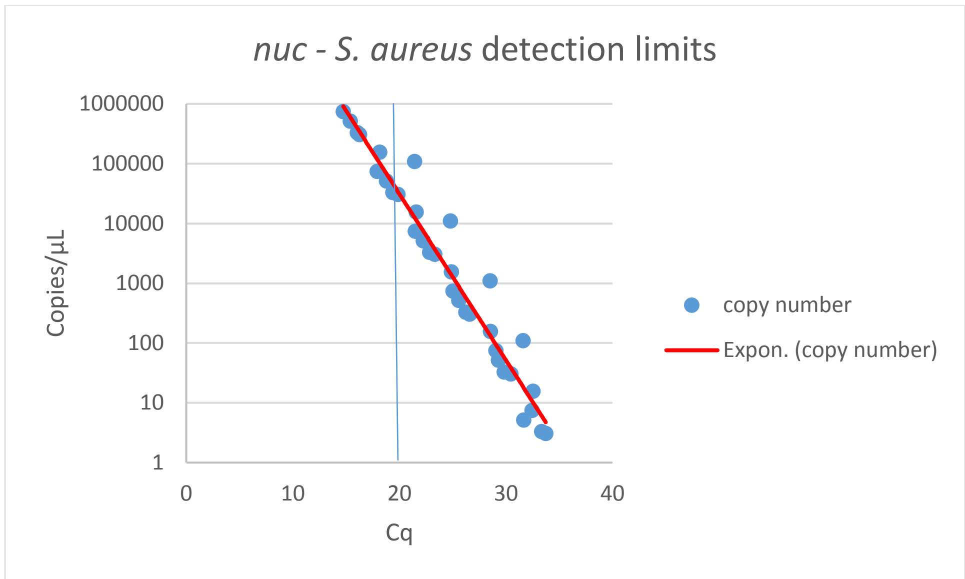

Figure 1: *nuc* – *S. aureus* detection limits of multiplex RT-PCR assay

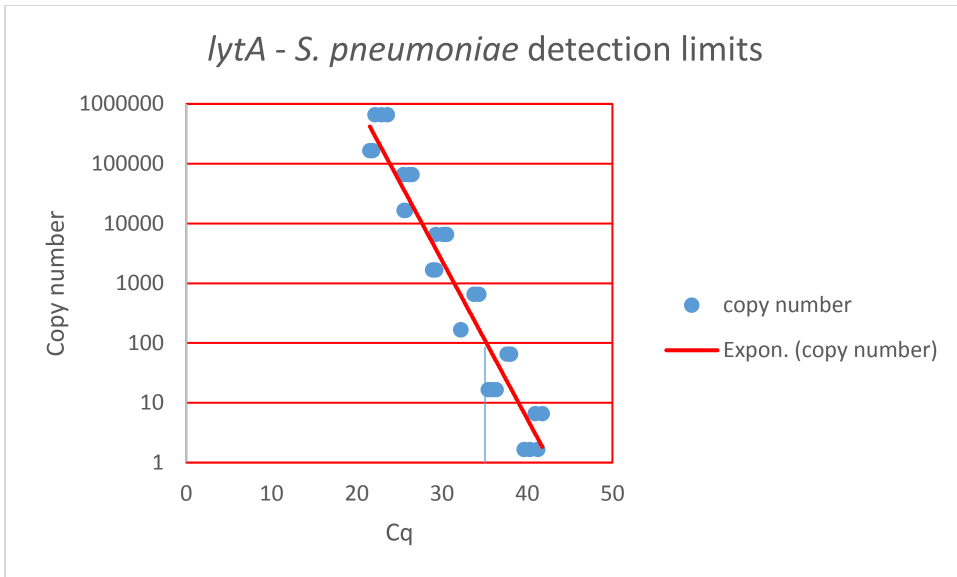

Figure 2: *lytA – S. pneumoniae* detection limits of multiplex RT-PCR assay

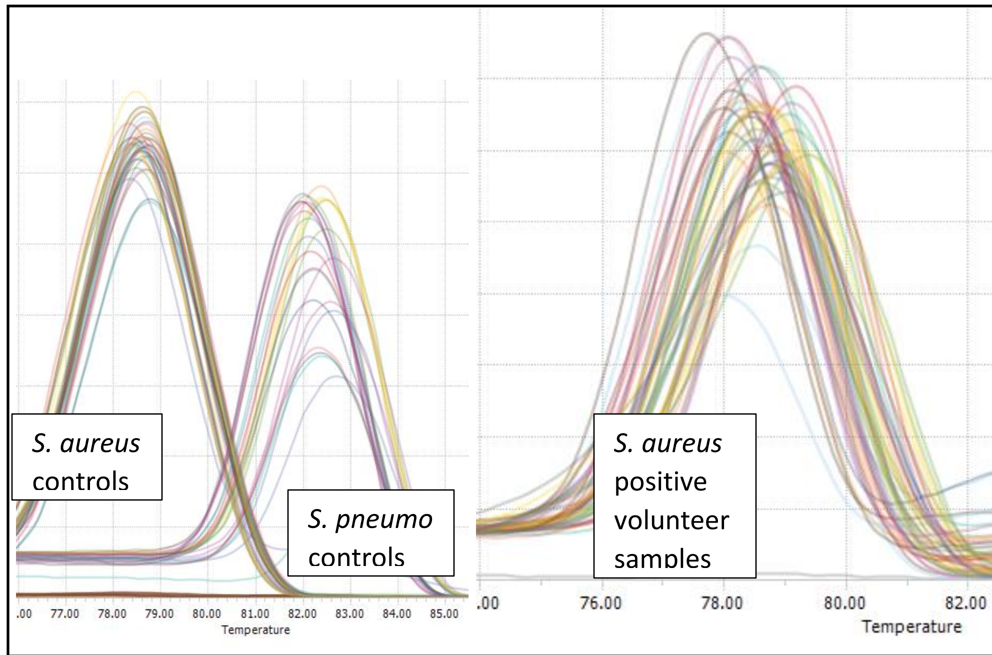

Figure 3: Multiplex melting peaks

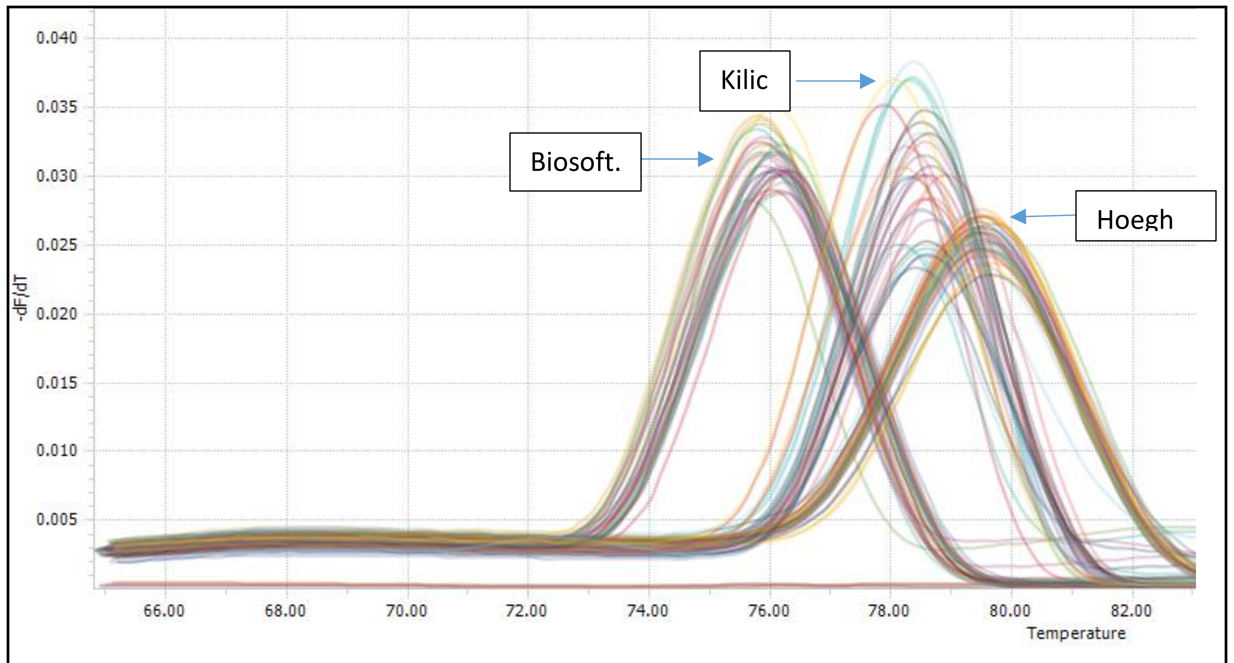

Figure 4: Melting peaks from *nuc* positive samples with three different primer sets in uniplex.

Appendix A: Institutional Review Board Approval Notice

Memorandum

TO: Paul Mann, Cathy Bammert, Josh Sharp, Yuba Gautam
Clinical Lab Science, Biology

DATE: August 13, 2015

FROM: Brian Cherry, Ph.D.
Assistant Provost/IRB Administrator

SUBJECT: IRB Proposal HS15-678
IRB Approval Dates: 8/13/2015- 8/13/2016**
Proposed Project Dates: 8/1/2015-8/1/2016
"Relationship between methicillin-resistant Staphylococcus aureus nasal colonization and vaccination with a pneumococcal vaccine in adults"

The Institutional Review Board (IRB) has reviewed your proposal and has given it final approval. To maintain permission from the Federal government to use human subjects in research, certain reporting processes are required.

A. You must include the statement "Approved by IRB: Project # HS15-678" on all research materials you distribute, as well as on any correspondence concerning this project.

B. If a subject suffers an injury during research, or if there is an incident of non-compliance with IRB policies and procedures, you must take immediate action to assist the subject and notify the IRB chair (dereande@nmu.edu) and NMU's IRB administrator (bcherry@nmu.edu) within 48 hours. Additionally, you must complete an Unanticipated Problem or Adverse Event Form for Research Involving Human Subjects

C. Please remember that informed consent is a process beginning with a description of the project and insurance of participant understanding. Informed consent must continue throughout the project via a dialogue between the researcher and research participant.

D. If you find that modifications of methods or procedures are necessary, you must submit a Project Modification Form for Research Involving Human Subjects before collecting data.

E. **If you complete your project within 12 months from the date of your approval notification, you must submit a Project Completion Form for Research Involving Human Subjects. If you do not complete your project within 12 months from the date of your

approval notification, you must submit a Project Renewal Form for Research Involving Human Subjects. You may apply for a one-year project renewal up to four times.

NOTE: Failure to submit a Project Completion Form or Project Renewal Form within 12 months from the date of your approval notification will result in a suspension of Human Subjects Research privileges for all investigators listed on the application until the form is submitted and approved.

All forms can be found at the NMU Grants and Research website:
http://www.nmu.edu/grantsandresearch/node/102

aw

Amanda Wigand

Graduate Assistant, Grants and Contracts

Northern Michigan University

906-227-2437

Appendix B: Volunteer questionnaire

Questionnaire to find relationship between MRSA and Prevnar Vaccination among adults:

Q1. Age

- o 18-30 years
- o 31-40 years
- o 41 -49 years
- o 50-59 years
- o 60-69 years
- o 75 years and above

Q2. Gender

- o Male
- o Female
- o Other

Q3. Race/Ethnicity

- o White
- o Hispanic or Latino
- o Black or African American
- o Native American or American Indian
- o Asian or Pacific Islander
- o Other

Q4. Have you ever had previous frequent skin infections?

- o Yes
- o No
- o Not Sure

Q5. Have you ever had previous frequent eye, nose, and throat infections?

- o Yes
- o No
- o Not Sure

Q6. Have you ever been previously diagnosed with *S. aureus* or MRSA infection?

- o Yes
- o No
- o Not Sure

Q7. Have you ever been previously diagnosed with *S. pneumoniae* infection?

- o Yes
- o No
- o Not Sure

Q8. Have you ever had history of cancer, bone marrow, organ transplant, or immunosuppressive condition?

- o Yes
- o No
- o Not Sure

Q9. Have you ever had history of recent or frequent hospitalization?

- o Yes

o No
o Not Sure

QI0. Have you ever been treated with antibiotics recently? If yes, what kind of antibiotics?

o Yes
o No
o Not Sure
List antibiotics: _ _ _ ______ _

Qll. Have you ever taken Pneumococcal conjugate (Prevnar) vaccination?

o Yes
o No
o Not Sure

Q12. Have you ever recently been prescribed antibiotics? What Type. ______ ,

o Yes
o No
o Not Sure

Q13. I have read the inform consent. I am willing to participate in this study and give consent for Nasal Swab.

Signature of the participant: ______________ _

Appendix C: Volunteer sample results

SID	Nostril	*nuc* pos (77.80°-79.20°C)	*lytA* pos (81.2°-88.5°C)	Cq	*S. pneumo* CX	*S. aureus* CX
15001	R	+	-	33.91	-	-
15003	R	+	-	34.01	-	-
15003	L	+	-	34.81	-	-
15004	L	+	-	34.74	-	-
15004	R	+	-	36.85	-	-
15005	R	+	-	15.81	-	-
15005	L	-	-	-	-	+
15006	L	+	-	33.60	-	+
15008	R	+	-	32.28	-	-
15008	L	-	-	-	-	-
15009	L	+	-	34.12	-	-
15009	R	+	-	35.38	-	-
15010	R	-	-		-	-
15010	L	+	-	35.83	-	-
15011	R	-	-	31.68	-	-
15011	L	+	-	34.64	-	-
15012	R	+	-	18.39	-	-
15012	L	+	-	19.76	-	-
15013	L	+	-	32.74	-	-
15013	R	+	-	34.08	-	-
15014	L	+	-	16.89	-	-
15014	R	+	-	19.49	-	+
15015	L	+	-	15.64	-	-
15015	R	+	-	16.19	-	-
15016	R	+	-	30.86	-	-
15016	L	+	-	30.95	-	-
15017	R	+	-	32.47	-	-
15017	L	+	-	12.75	-	+
15018	L	+	-	33.22	-	-
15018	R	-	-	33.45	-	-
15019	L	+	-	14.47	-	+
15019	R	+	-	14.59	-	+
15020	R	+	-	13.40	-	+
15020	L	+	-	13.60	-	+
15021	R	+	-	32.71	-	-
15021	L	+	-	34.40	-	-

SID	Nostril	*nuc* pos (77.80°-79.20°C)	*lytA* pos (81.2°-88.5°C)	Cq	*S. pneumo* CX	*S. aureus* CX
15022	L	+	-	31.87	-	-
15023	R	+	-	32.77	-	-
15024	R	+	-	30.40	-	-
15024	L	+	-	36.06	-	-
15025	L	+	-	33.15	-	-
15025	R	+	-	32.35	-	+
15026	R	+	-	32.93	-	+
15026	L	+	-	35.37	-	+
15027	L	+	-	34.49	-	-
15027	R	+	-	36.26	-	-
15028	L	+	-	33.81	-	-
15028	R	+	-	35.24	-	-
15029	L	+	-	32.86	-	-
15029	R	+	-	34.70	-	-
15031	L	+	-	17.27	-	+
15031	R	+	-	14.20	-	-
15032	R	+	-	33.87	-	-
15032	L	+	-	35.63	-	+
15033	R	-	-	29.31	-	-
15033	L	+	-	32.58	-	-
15034	L	+	-	33.54	-	-
15034	R	+	-	34.24	-	-
15035	L	+	-	29.50	-	-
15035	R	+	-	30.55	-	-
15036	L	+	-	33.79	-	-
15036	R	+	-	34.23	-	-
15037	L	+	-	15.93	-	+
15037	R	+	-	16.29	-	+
15038	R	+	-	14.98	-	-
15039	R	+	-	16.48	-	+
15039	L	+	-	31.52	-	+
15040	R	+	-	18.14	-	-
15040	L	+	-	32.00	-	-
15041	R	+	-	33.62	-	-
15041	L	+	-	35.60	-	-
15042	R	+	-	33.60	-	-
15042	L	+	-	34.46	-	-

SID	Nostril	*nuc* pos (77.80°-79.20°C)	*lytA* pos (81.2°-88.5°C)	Cq	*S. pneumo* CX	*S. aureus* CX
15044	L	-	+	33.20	+	-
15045	R	-	+	30.44	-	-
15045	L	+	-	32.06	-	-
15046	L	+	-	27.12	-	-
15046	R	+	-	29.68	-	-
15047	L	-	+	30.22	+	-
15047	R	+	-	32.83	-	-
15048	R	+	-	33.97	-	-
15048	L	+	-	35.83	-	-
15049	L	+	-	13.49	-	+
15049	R	+	-	15.25	-	+
15050	R	-	+	34.24	-	-
15050	L	+	-	34.91	-	-
15051	L	+	-	32.52	-	-
15051	R	+	-	35.72	-	-
15052	L	+	-	35.74	-	-
15052	R	+	-	29.87	-	-
15053	L	+	-	33.26	-	-
15053	R	+	-	35.28	-	-
15054	L	+	-	31.39	-	-
15054	R	-	+	33.85	-	-
15055	L	+	-	32.85	-	-
15055	R	+	-	36.01	-	-
15056	L	+	-	33.70	-	-
15057	R	+	-	14.09	-	+
15057	L	+	-	16.97	-	+
15058	L	+	-	26.85	-	-
15059	R	+	-	30.91	-	-
15059	L	+	-	33.37	-	-
15060	L	+	-	32.40	-	-
15060	R	+	-	33.93	-	-
15061	L	+	-	15.14	-	+
15061	R	+	-	15.39	-	+
15062	R	+	-	15.03	-	-
15062	L	+	-	16.00	-	-
15063	R	+	-	27.66	-	-
15063	L	+	-	33.84	-	+

SID	Nostril	*nuc* pos (77.80°-79.20°C)	*lytA* pos (81.2°-88.5°C)	Cq	*S. pneumo* CX	*S. aureus* CX
15064	R	+	-	31.28	-	-
15064	L	+	-	33.06	-	-
15065	R	+	-	21.81	-	-
15065	L	+	-	33.23	-	-
15066	R	+	-	33.91	-	-
15066	L	+	-	36.17	-	-
15068	L	+	-	31.56	-	-
15068	R	+	-	33.32	-	-
15069	L	+	-	33.49	-	-
15069	R	-	-	35.12	-	-
15071	L	+	-	13.68	-	-
15072	R	+	-	32.77	-	-
15073	L	+	-	30.46	-	-
15074	5L	+	-	34.06	-	-
15074	R	+	-	34.33	-	-
15074	L	-	-	-	-	-
15075	L	-	-	36.11	-	-
15076	L	+	-	27.69	-	-
15076	R	+	-	31.52	-	-
15077	R	+	-	31.16	-	-
15077	L	+	-	35.19	+	-
15078	R	+	-	32.35	-	-
15078	L	+	-	34.90	-	-
15079	L	+	-	36.56	-	-
15079	R	-	-	-	-	-
15080	L	+	-	31.48	-	-
15080	R	-	-	-	-	-
15082	R	+	-	30.76	-	-
15082	L	+	-	32.19	-	+
15083	L	+	-	19.02	-	-
15084	L	+	-	31.25	-	-
15085	L	+	-	29.66	-	-
15087	L	+	-	29.00	-	-
15088	L	+	-	10.87	-	-
15090	L	-	+	28.24	-	-
15099	L	+	-	17.03	-	-
15099	R	+	-	18.98	-	-

SID	Nostril	*nuc* pos (77.80°-79.20°C)	*lytA* pos (81.2°-88.5°C)	Cq	*S. pneumo* CX	*S. aureus* CX
15100	L	+	-	35.95	-	-
15100	R	-	-	-	-	-
15101	L	+	-	29.48	-	-
15101	R	+	-	36.21	-	-
15102	L	+	-	28.21	-	-
15102	R	+	-	35.77	-	-
15103	L	+	-	28.62	-	-
15103	R	+	-	34.93	-	-
15104	L	+	-	28.95	-	-
15104	R	+	-	35.37	-	-
15106	L	+	-	12.86	-	-
15107	L	+	-	12.85	-	-
15108	L	+	-	13.16	-	-
15109	L	+	-	27.21	-	-
15111	L	+	-	13.60	-	-
15112	L	+	-	28.13	-	-
15113	L	+	-	27.65	-	-
15115	L	+	-	26.22	-	-
15117	L	+	-	29.12	-	-
15118	L	+	-	27.65	-	-
15119	L	+	-	26.09	-	+
15120	L	+	-	27.52	-	-
15123	L	+	-	11.08	-	+
15124	L	+	-	24.21	-	-
15125	L	+	-	10.40	-	+
15126	L	+	-	9.93	-	+
15127	L	+	-	28.31	-	-
15128	L	+	-	25.85	-	-
15129	L	+	-	10.96	-	+
15130	L	+	-	13.41	-	+
15131	L	+	-	23.62	-	+
15132	L	+	-	23.99	-	-
15133	L	+	-	25.63	-	-
15150	L	+	-	15.21	-	+
15151	L	+	-	28.84	-	-
15152	L	-	+	28.66	-	-
15153	L	+	-	13.63	-	-

SID	Nostril	*nuc* pos (77.80°-79.20°C)	*lytA* pos (81.2°-88.5°C)	Cq	*S. pneumo* CX	*S. aureus* CX
15154	L	+	-	13.54	-	+
15155	L	+	-	28.12	-	-
15156	L	+	-	30.87	-	-
15157	L	+	-	11.95	-	+
15158	L	+	-	28.76	-	-
15159	L	+	-	26.70	-	-
15160	L	+	-	14.57	-	-
15161	L	+	-	29.27	-	-
15162	L	+	-	26.76	-	-
15163	L	+	-	12.77	-	-
15164	L	+	-	29.09	-	-
15165	L	+	-	28.15	-	-
15166	L	+	-	28.09	-	-
15167	L	+	-	13.43	-	+
15169	L	+	-	13.30	-	+
15170	L	+	-	29.15	-	-
15171	L	+	+	26.49	-	-
15172	L	+	-	11.60	-	+
15173	L	+	-	29.68	-	-
15174	L	+	-	14.07	-	+
15175	L	+	-	28.70	-	-
15176	L	+	-	13.35	-	+
15177	L	+	-	28.72	-	-
15178	L	+	-	12.82	-	+
15179	L	+	-	26.74	-	-
15180	L	+	-	28.76	-	-
15181	L	+	+	29.44	-	-
15182	L	+	-	29.56	-	-
15183	L	+	-	28.97	-	-
15184	L	-	-	-	-	-
15185	L	+	-	29.19	-	-
15186	L	+	-	26.57	-	-
15187	L	+	-	27.64	-	-

Appendix D: Uniplex primer evaluation of *nuc* positive multiplex samples

SID	Nostril	Multiplex Cq	Kilic *nuc* Cq	Hoegh *nuc* Cq	Biosoft *nuc* Cq
15126	L	9.93	10.38	11.24	21.43
15125	L	10.40	11.97	11.56	10.62
15088	L	10.87	11.39	12.11	11.69
15129	L	10.96	11.14	12.37	11.80
15123	L	11.08	12.87	12.11	11.50
15172	L	11.60	12.12	12.75	12.27
15157	L	11.95	12.70	12.85	12.24
15163	L	12.77	13.36	13.68	15.37
15178	L	12.82	13.15	13.88	12.90
15107	L	12.85	13.29	13.64	12.43
15107	L	12.85	14.00	14.13	13.54
15106	L	12.86	13.31	13.96	16.13
15108	L	13.16	13.64	14.57	14.16
15169	L	13.30	14.41	14.89	14.97
15176	L	13.35	13.43	14.22	16.72
15130	L	13.41	13.13	14.00	13.86
15167	L	13.43	14.14	14.08	13.12
15154	L	13.54	13.79	14.77	14.00
15111	L	13.60	13.58	14.28	13.65
15174	L	14.07	14.27	15.09	14.41
15019	L	14.47	35.68	35.67	-
15160	L	14.57	14.83	15.48	17.95
15038	R	14.98	15.51	16.28	19.94
15150	L	15.21	32.60	32.25	33.27
15040	R	18.14	18.21	18.73	18.26
15012	L	18.39	20.04	20.32	-
15083	L	19.02	19.78	19.87	19.95
15132	L	23.99	25.04	25.36	26.10
15171	L	26.49	27.73	27.46	26.48
15186	L	26.57	26.22	27.04	27.84
15179	L	26.74	27.70	28.96	26.06
15162	L	26.76	27.64	27.76	30.44
15120	L	27.15	28.60	29.16	-
15109	L	27.21	28.36	28.00	26.48
15118	L	27.65	28.25	28.52	27.14
15155	L	28.12	28.94	28.29	28.10
15112	L	28.13	29.02	28.56	-
15102	L	28.21	29.13	28.54	28.10
15087	L	29.00	28.66	29.91	-
15164	L	29.09	29.60	29.45	29.35
15117	L	29.12	31.10	30.16	28.72

SID	Nostril	Multiplex Cq	Kilic *nuc* Cq	Hoegh *nuc* Cq	Biosoft *nuc* Cq
15161	L	29.27	28.04	27.89	28.23
15101	L	29.48	30.29	29.43	29.75
15173	L	29.68	30.11	29.24	28.73
15077	L	31.16	35.56	35.93	-
15084	L	31.25	31.53	-	30.08
15054	R	31.39	34.14	32.29	-
15080	L	31.48	31.61	31.91	-
15103	L	34.00	28.86	28.44	-

Appendix E: MSA sub-culture results

SID	Initial sample results				MSA Sub-culture results	
	Nostril	TM1 (°C)	Cq	Culture result	MSA sub-culture	RT-PCR
15150	L	78.42	15.21	*S. aureus* pos	*S. aureus*	
15151	L	78.62	28.84	*S. aureus* neg	non-*aureus Staph.*	negative
15159	L	79.09	26.70	*S. aureus* neg	no growth	
15160	L	78.27	14.57	*S. aureus* neg	*S. aureus*	
15163	L	78.51	12.77	*S. aureus* neg	*S. aureus*	
15166	L	78.58	28.09	*S. aureus* neg	no growth	
15175	L	78.60	28.70	*S. aureus* neg	no growth	
15180	L	78.69	28.76	*S. aureus* neg	non-*aureus Staph.*	negative
15181	L	78.26	29.44	*S. aureus* neg	non-*aureus Staph.*	negative
15182	L	78.49	29.56	*S. aureus* neg	no growth	
15183	L	78.79	28.97	*S. aureus* neg	non-*aureus Staph.*	negative
15185	L	78.56	29.19	*S. aureus* neg	non-*aureus Staph.*	negative
15186	L	78.52	26.57	*S. aureus* neg	non-*aureus Staph.*	negative