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Expression Levels of Virulence Genes in Group A Streptococci: A Response to Aerosolized Propylene Glycol

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EXPRESSION LEVELS OF VIRULENCE GENES IN GROUP A STREPTOCOCCI:
A RESPONSE TO AEROSOLIZED PROPYlene GLYCOL

by

MICHAEL S. COSTELLO

A thesis submitted in partial fulfillment of the requirements for the Honors in the Major Program in Biology in the College of Sciences and in the Burnett Honors College at the University of Central Florida Orlando, Florida

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Thesis Co-Chairs: Eric Hoffman, Ph.D. and Sean Moore Ph.D.
Abstract

Electronic cigarette usage is becoming increasingly prevalent among school age children and young adults. A known bactericidal agent, propylene glycol, is often used as a carrier for nicotine, flavoring, and additional constituents of electronic cigarette juice. This study examined the relationship between propylene glycol and virulence gene expression in *Streptococcus pyogenes*, a respiratory tract pathogen commonly found in school-age individuals. A variety of virulence genes controlled by the three stand alone regulators mga, RofA, and Rgg/RopB were sampled in an effort to understand the pathway by which virulence is affected. The genes chosen encode C5a peptidase, fibronectin binding protein, hyaluronate lyase, NAD glycohydrolase, Streptococcal pyrogenic exotoxin A and B, streptodornase, streptokinase, Streptolysin O, and Streptolysin S. No significant change in gene expression was observed, but a novel method to test the effects of aerosols on cells was developed. This method can be used in the future to observe the effect of aerosols, including commercial electronic cigarette juice, on both bacterial and mammalian cells.
Dedication

Yeah, this thesis is dedicated to all the teachers that told me I'd never amount to nothing, to all the people that lived above the buildings that I was studying in front of, that called the police on me when I was just trying to make some money to feed my Boston Terrier.

And all the people in the struggle, you know what I'm saying?
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Introduction

Streptococcus pyogenes (Group A Streptococcus) is a Gram-positive bacterial species that exclusively colonizes humans, and is responsible for 600 million infections and 500,000 deaths each year.\(^1\) M protein serotypes of Group A Streptococci (GAS) are typically found in the nasopharynx and is the most common cause of bacterial pharyngitis, where school age children ages 5 to 15 years old are the most frequently affected.\(^2\) Group A streptococcal infections are also responsible for debilitating diseases such as toxic shock syndrome and necrotizing fasciitis, albeit these diseases are far more infrequent and are associated with different serotypes of Group A streptococcus.\(^2\)

GAS is an effective pathogen due to its highly evolved and complex virulence systems, which rely on stand-alone transcriptional regulators such as mga and two component regulatory regions like CovR/S to alter gene expression in various environmental conditions.\(^3\) GAS is not considered normal flora, but can persist in the nasopharynx without displaying clinical symptoms of disease. However, environmental stress imposed by the host to eradicate the infection often elicits a regulatory response by GAS, prompting changes in virulence gene expression.\(^3\) These genes code for proteins (virulence factors) that facilitate colonization, infection, immune evasion, and long term persistence in the host.\(^4\) The highly regulated nature of virulence gene expression\(^4,\,5\) indicates a fitness cost associated with their expression, and suggests that in the absence of stress, the expression of these genes would be disadvantageous for the pathogen.\(^5\) This makes the analysis of virulence gene transcription levels in GAS
an effective method to measure pathogenic stress response to extracorporeal influences.

Electronic cigarette use has increased markedly among school age children and adults in the last two years. According to the National Youth Tobacco Survey in 2014, the percentage of high school students in the United States who currently use electronic cigarettes has grown from 4.5% in 2013, to 13.4% in 2014, and usage among middle school students has also more than tripled from 1.1% in 2013 to 3.9% in 2014. From 2010 to 2013 current electronic cigarette use among adults in the United States has increased 1% to 2.6%. This unprecedented growth in popularity has intensified the disparity between the what is known about the effects of electronic cigarettes on humans, and what is required to make informed decisions regarding e-cigarette use. It is especially troubling that electronic cigarette use has increased 3-fold among children that are statistically most likely to incur a GAS infection.

Electronic cigarettes have disposable or refillable cartridges containing a viscous liquid (e-liquid), which is often comprised of water, nicotine, propylene glycol, vegetable glycerin, flavoring agents, ethanol, ammonia solutions, nicotine essence, and the antioxidant t-butoxytoulene. This mixture is aerosolized using a portable atomizer at approximately 55 °C and is inhaled by the user.

Propylene glycol (PG) is of particular interest due to its effectiveness as a bactericidal agent. PG is a short chain alcohol used to solubilize nicotine, and is the primary component of e-liquid, often referred to as the base. The percent composition of propylene glycol in the e-liquid is largely dependent on the manufacturer, but it is
prevalent in most juices (more than 50% v/v). According to Offermann, the hazard quotient of each major chemical constituent of e-liquid can be calculated as the exposure in micrograms per cubic meter divided by the NSRL (No Significant Risk Level) and CREL (Chronic Reference Exposure Level) guideline for that compound.\textsuperscript{12} Propylene glycol ranked highest among all major e-liquid components with a hazard quotient of 967, and nicotine ranked second highest at 222.\textsuperscript{12}

Previous studies by Robertson and Puck have aerosolized bacteria and treated them with an aerosolized concentration of PG that is significantly higher than any commercial e-cig can provide, in conditions that do not resemble the nasopharynx.\textsuperscript{9, 10} This killed most gram positive bacteria, but it is possible that under more mild conditions, GAS may be more fit to mount a stress response after prolonged exposure to PG.

Several antimicrobial compounds have been demonstrated to activate a global virulence regulator, CovR/S. An anti-biofilm agent, 3-furancarboxaldehyde,\textsuperscript{13} sub-inhibitory doses of clindamycin,\textsuperscript{14} and the human antimicrobial peptide LL-37\textsuperscript{15} are among those demonstrated to elicit a regulatory response. It is hypothesized that the antimicrobial nature of PG may activate CovR/S in a similar manor. This study explores the avenues by which GAS organisms alter virulence gene expression in response to the extracorporeal pressure presented by aerosolized propylene glycol.
Literature Review

**Serotype Selection**

P Sumby and JJ Ferretti first sequenced the genome of Streptococcus *pyogenes* strains MGAS5005 and SF370. These are the two of most prominent serotypes isolated in clinical settings.\(^{16,17}\) From the early 1920's until 1987, SF370-like strains were predominantly isolated in clinical settings, but from 1987 to 1989, were almost completely displaced by MGAS5005-like strains.\(^{18}\) In an effort to understand the genetic diversification event leading to the strong reemergence of *S. pyogenes* as a worldwide threat, 3615 strains of serotype Emm protein 1 (M1) Group A Streptococcus were sequenced by Nasser and colleagues in 2014 and compared to the reference serotypes MGAS5005 and SF370. Using next generation sequencing and molecular phylogenetics, it was determined that MGAS5005 most likely incurred a horizontal gene transfer event as a result of phage acquisition encoding for the Streptolysin O and NAD glycohydrolase virulence genes.\(^{18}\) As a result the fitness landscape shifted in favor of MGAS5005. The MGAS5005 strain has proven to be more virulent than SF370 in multiple studies, and has most likely contributed to its global ascendancy.\(^{18-20}\) The sheer dominance of the contemporary MGAS5005 – like strains makes them an excellent serotype to study because their robust pathogenic qualities may allow them to persist in the presence of a propylene glycol antagonist. ATCC 19615 is a Streptococcus *pyogenes* Group A beta-hemolytic reference strain isolated from a patient’s throat. When aligned with MGAS5005, it produces a 99 percent identity, and a 91 percent query cover according to the NCBI BLAST algorithm.\(^{21}\) ATCC 19615 produces the same
virulence factors as MGAS5005 and regulates transcription in an identical manor, and has been chosen as the subject of this study.

**Regulation of Virulence Gene Expression**

To persist in its host, *S. pyogenes* must have the ability to control gene expression in response to host immune pressure and environmental stress.\(^3\)\(^4\) GAS organisms rely on a combination of stand-alone regulatory regions, two-component regulatory systems, quorum sensing to change virulence gene expression.\(^4\)

**Stand-Alone Regulons**

GAS organisms rely on three well characterized “stand-alone” regulatory systems to effect change in virulence gene expression, and each rises to prominence during different growth phases.\(^4\)

Mga (multiple gene activator) is a transcriptional activator often associated with colonization due to its strong expression profile during exponential phase and because the majority of the genes under its control are responsible for the production of proteins that promote adhesion, internalization, and immune evasion.\(^4\) Mga directly binds the promoter of genes under its control. These genes include *emm* (M-protein), *sof1* (serum opacity factor), *ScpA* (C5a Peptidase), and *sic* (secreted inhibitor of complement). *Mga* is also responsible for its own regulation, but can be repressed by *RotA* and *RopB* operons.\(^22\)\(^23\) Environmental factors that result in positive activation of *mga* include increased temperature, increased carbon dioxide concentration, and iron limiting conditions.\(^24\)\(^25\)
RofA like proteins (RALPs) are another DNA binding subclass of stand-alone transcriptional regulators. RofA is involved in autoregulation, positive regulation of sfb1 (fibronectin-binding protein), and negative regulation of mga, SpeA (Super antigen), SpeB (Cysteine protease), and sagA (hemolysin).26-28 The pattern of regulation dictated by RALPs and their expression maxima during stationary phase suggest RofA like proteins functions to shift S. pyogenes from colonization to persistence (exponential growth phase to early stationary phase) in its host. Environmental pressures that affect RALP expression include oxidative stress, and increased incubation temperature.29, 30 VanHeyningen and colleagues demonstrated a significant increase in sfb1 in the when grown in media containing 1mM potassium ferricyanide.31 RofA is susceptible to down-regulation under oxidative stress (positive redox potential) presented by potassium ferricyanide, resulting in an up-regulation of sfb1.

Rgg/ RopB is a regulatory region associated with increased expression of the cysteine protease SpeB, though it is thought to have an indirect role, acting through existing regulatory networks. Rgg/RopB has been demonstrated to up-regulate CovR/S, ihk/irr, and fasBCAX, while down-regulating mga.32
Two-Component Transduction Systems (TCS)

Sequencing the genome of M1 GAS revealed an average of 13 two-component signal transduction systems per cell.\(^{17}\) Since then, only 3/13 regulons have been characterized, and the remaining have been assigned function on the basis of sequence homology.\(^4\) Two component transduction systems (TCS) are typically comprised of a membrane bound sensory protein and a cytoplasmic response regulator.\(^{33}\) The autophosphorylation of a histidine kinase domain in response to an extracellular signal encourages the transfer of a phosphoryl group to a regulatory protein. This changes the regulator’s binding affinity for its target promoter regions, altering the transcription frequency of a variety of genes.\(^{33}\)

*FasBCAX* is a TCS dependent on growth cycle and plays a role in the upregulation *saga* and *ska* in late-exponential phase. An untranslated regulatory RNA (*fasX*) mediates the cytoplasmic response.\(^4\) The expression of FasBCAX results in tissue destruction via host cell lysis or apoptosis.

The most dominant TCS in terms of expression influence is *CovR/S* (Cluster of Virulence), with 15% of all chromosomal genes being affected by its activation.\(^4\) As with the regulatory pathways mentioned above, *CovR/S* is highly dependent on growth phase, and is most active during early stationary phase. It positively regulates *hasABC* (Capsule), and negatively regulates *ska, sagA, sda, SpeB*, and additional secreted virulence factors.\(^{34, 35}\)
Exposure to the anti-biofilm agent 3-furancarboxyaldehyde prompts a down-regulation of CovR (Cluster of Virulence Repressor), resulting in the positive regulation of emm genes including C5a peptidase (ScpA), secreted inhibitor of complement (sic), and streptodornase (sda), Streptolysin S (sagA), and Streptokinase.\textsuperscript{13} (Figure 2)

Because the molecular mechanism behind the antimicrobial nature of propylene glycol has not been elucidated, virulence genes under the control of various regulatory domains were chosen as a subject of this study. Inferences can be drawn about the molecular mechanism based on the trends in transcription of virulence genes. GAS demonstrates susceptibility to different antimicrobial compounds that affect transcription levels of regulatory regions, and these studies may provide a clue to GASs response to propylene glycol \textit{in vitro}.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{CovR Regulon. Green arrows indicate up-regulation and red arrows indicate down-regulation. Adapted from "Virulence factor regulation and regulatory networks in Streptococcus pyogenes and their impact on pathogen–host interactions." \textit{Trends In Microbiology} [serial online]. January 1, 2003;11:224-232. Available from: ScienceDirect, Ipswich, MA. Adapted with permission.}
\end{figure}
Virulence Factor Function

Immune Evasion and Toxic Shock Syndrome

It has been reported that streptococcal chronic carriage and treatment failure can be explained partly by the function of Streptolysin O (SLO) and its co-toxin NAD-glycohydrolase (NADase). SLO is an oxygen-labile hemolytic cytolysin that is lytic towards any cell wall containing cholesterol. The mechanism details pore formation in host cells mediated by SLO, and the translocation of NADase into the cytosol. This prevents maturation of GAS-containing autophagosomes, resulting in an increased intracellular survival time throughout xenophagy.\textsuperscript{36}

Streptolysin S (sagA) is a more robust hemolysin due to both its stability in the presence of atmospheric oxygen, small size, and highly modified nature, where the latter two are responsible for its non-antigenic property.\textsuperscript{37}

Another evasion tactic by GAS includes the production of C5a peptidase (ScpA) on the cell wall surface. ScpA is responsible for cleaving the C5a complement from Component 5, which is necessary for neutrophil recruitment.\textsuperscript{38}

Streptococcal pyrogenic exotoxin A (SpeA) is a superantigenic protein that binds T-cell receptors and antigen presenting cells on major histocompatibility complex II, activating T-Cell production of cytokines. The surge in cytokine concentration is associated with organ failure and streptococcal toxic shock syndrome (STSS). Streptococcal pyrogenic exotoxin B is a secreted cysteine protease capable of cleaving immunoglobulins.\textsuperscript{39}
**Adherence, Internalization, and Necrotizing Fasciitis**

GAS binds and permeates host soft tissue with a plethora of virulence factors. Fibronectin binding protein (*Sfb1*) is required for adherence and invasion of respiratory epithelial cells as demonstrated by antibody blocking of *sfb1*.40

GAS is capable of exploiting host machinery to facilitate systemic spread through the degradation of tissue barriers. Streptokinase (*ska*) is an example of plasminogen activating protein. A plasminogen activator complex is formed between ska and plasminogen in a stoichiometric fashion, and results in an active site within the bound plasminogen structure. The activation bond is cleaved to produce plasmin.41 Plasmin is a serine protease required to digest fibrin clots. It is also necessary for the activation of metalloproteases and collagenases necessary for tissue repair and remodeling.41

Streptodornase (*sda*) is a phage-acquired DNase that also participates in host tissue destruction through hydrolysis of the phophodiester backbone of nucleic acids.

Hyaluronate lyase (*hyal*) is a spreading factor responsible for cleaving hyaluronan, a staple of the soft connective tissue extracellular matrix. Soft connective tissue matrices are comprised of B-linked N-acetylglucosamine and glucuronic acid, which are hydrolyzed by a hyaluronate lyase catalyzed elimination reaction.42 The breakdown of tissue barriers allows GAS to spread systemically throughout the host.
Propylene Glycol and Group A Streptococci

Robertson and Puck have extensively explored (1941-1943) the bactericidal nature of aerosolized propylene glycol with respect to streptococcus pyogenes in vitro. They concluded that the bactericidal action of PG was greatest at a moderate humidity (58%), low temperature (25 °C), and when the bacterial colonies were aerosolized along with the PG at the saturation level for PG. They found the bactericidal action of PG to be significantly less effective at higher relative humidities and temperatures alike. Humidity like that experienced in the nasopharynx in humans produces a lower saturation limit for PG according to Raoult’s law, and is most likely responsible for a lower bactericidal rate. To replicate the action of PG on bacterial species that colonize the throat, growth conditions include a temperature of 37 °C, a high relative humidity that mimics the respiratory tract (>98%), and plating on solid media to facilitate a biofilm. Alternative studies have focused on the antimicrobial effects of PG in broth media, and have found it an effective bactericidal against GAS in vitro.

RT-qPCR

The majority of GAS virulence factors are proteins and quantification requires multiple primary antibodies, making it a challenging and costly option. With the advent of quantitative thermocyclers, it is possible to measure transcriptional levels of genes using reverse transcriptase to convert mRNA gene transcripts to cDNA. cDNA is generated via random hexamer priming, where randomly generated oligonucleotides (6 nt long primers) are introduced into a total RNA extract with a reverse transcription
enzyme and free NTPs, resulting in amplification and conversion of RNA to cDNA. This cDNA library serves as the template for resultant qPCR reactions.

A primer set can be used to amplify the cDNA with a DNA polymerase and free dNTPs, much like traditional PCR. The caveat being that an intercalating dye is also present in the reaction mixture, and binds to product as it is made, emitting a fluorescent signal. After each cycle, the fluorescence is measured, and should increase in proportion to the amount of product. The first cycle in which the fluorescence signal becomes distinguishable among background fluorescence is referred to as the Ct or Cq. The transcript that reaches this threshold most quickly is said to have been initially present in a larger relative frequency.

To compare Ct values between experimental groups, the assay must be normalized to an endogenous gene or RNA spike to account for variable extraction and cDNA conversion efficiencies that likely occur. An endogenous gene (Housekeeping Gene) is a gene (typically required for cell function) whose expression pattern remains constant despite environmental stress. The transcription of this gene provides an internal reference to compare the up-regulation or down-regulation of target genes, providing an accurate depiction of transcriptional change.\(^{45}\) The problem with normalization to a housekeeping gene is that while the gene may have been transcribed in a consistent manor under one set of experimental conditions, it is susceptible to erratic behavior under different conditions.\(^{46}\) Therefore, it is undesirable to assume a housekeeping gene will have a stable expression profile under novel conditions where its expression has not been characterized. A RNA spike of a known concentration and
identity can be used to both evaluate the efficiency of RNA extraction and cDNA conversion, and can also serve as a normalization tool. It is also possible to use a RNA spike solution that hasn’t been quantified if it is homogenous. Appropriate steps must be taken to ensure this spike is in equal proportions between samples. An effective way is to perform a total protein quantification assay with Bradford reagent to compensate for different lysis efficiencies. The total protein in the cell should be constant and independent of individual shifts in protein expression. Once RNA concentration has been normalized to total protein, the spike can be used as a reference.

The transcription of mRNA does not necessarily mean the protein will be translated, and sometimes is degraded before reaching the ribosome. However, this can be accounted for by designing the forward primer in a way that it anneals to or upstream of the Shine-Dalgarno region. This region is typically found 8 bases upstream of the start codon, and functions as the ribosomal binding site for translation. This ensures that only transcripts projected for translation are being accounted for.

**Arduino**

Arduino is an open-source programming platform that provides an affordable way to create scientific instruments, namely turning sensor inputs into electrical outputs utilizing an integrated development environment (programming software) that mimics C language, and a microcontroller. The versatility presented by Arduino technology is ideal for customizing a chamber to test the effects of an aerosol on bacteria. It allows for the ability to record time, the relative concentration of propylene glycol, temperature, and relative humidity. It also has the ability to turn on a solid-state relay controlling an
external power source to supply an electronic cigarette, with the ability to control puff duration and interval between puffs.
Methodology

Bacterial Strain and Media

*Streptococcus pyogenes* ATCC 19615 is a Lancefield’s group A beta-hemolytic reference strain originally isolated from a child’s throat. It was obtained from an institution stock and was grown in aerobic conditions at 37 °C on Blood Agar (TSA + 5% Sheep’s blood).

Apparatus Design

Hardware

A few factors were taken into consideration to create a chamber suitable for housing bacteria and an electronic cigarette. The glass fish tank design serves two practical purposes, one being that it mitigates the risk of a fire hazard while working with a resistive heating element, and the second being that the environment can be monitored without disruption. The latter is important because *S. pyogenes* has shown the ability to degrade mRNA corresponding to certain virulence factors by 50% in 5 minutes or less. The tank contains three chambers, isolated by glass dividers and sealed with silicone. The experimental chamber containing the electronic cigarette and propylene glycol was designated to the far right, and the control chamber in the absence of propylene glycol was placed on the far left, leaving the middle chamber to house the Arduino and Crydom solid state relay (San Diego, CA). The sensor wires were channeled through a hole in each divider bored by diamond dusted drill bits. The remaining gap was filled with silicone.
Figure 2: Schematic of Chamber

Figure 3: Photo of Chamber
Figure 4: Arduino wiring diagram. The load depicted in the solid-state relay circuit is representative of the resistive heating element from the electronic cigarette.

The microcontroller used was an Arduino clone (Sainsmart Uno R3), used in parallel with two Sainsmart MQ-3 Gas sensors, which have a Tin-Dioxide membrane that decreases in the presence of alcohol gas, resulting in a higher voltage reading. This voltage reading can be correlated with a specific concentration of propylene glycol, but this can be challenging as the reading varies as a function of temperature and humidity, and concentration in PPM would not be worthwhile data to report. Instead, both a 12 minute average time between puffs and average inhale time of 4.2 were chosen based on survey of 3,587 participants.49

The MQ-3 sensors serve to show trends in concentration and validate electronic cigarette function, and also provide evidence that the control was untreated throughout
the trial. A DH22 digital temperature and relative humidity sensor (Adafruit Industries New York, NY) was also placed in the experimental chamber to ensure relative humidity closely mimicked that of a respiratory airway \((x > 98\% \text{ RH})\).\(^{43}\)

The electronic cigarette was obtained from eSmokerOnline (Orlando, FL), and is considered a “genesis style atomizer” with a stainless steel wicking system. The stainless steel wick (#500 gauge obtained from eSmokerOnline) was rolled tightly into a cylinder and oxidized with a flame where it met the atomizer to avoid a short circuit. A 28-gauge nichrome wire with a resistance of 1.66 ohms was wrapped tightly around the stainless steel wick, from the negative to positive post on the atomizer. The electronic cigarette was powered by a generic 5V regulated wall source with a 3A current flow dictated by the 1.66 ohm nichrome resistor coil, according to Ohm’s Law.

**Software**

Using the Arduino IDE, a program was designed so that the three sensors were read simultaneously and logged the data to a SD card alongside the elapsed time. After 12 minutes, the Arduino was programmed to turn on a digital pin emitting a current of 4 mA, closing the solid-state relay circuit. The current was supplied to the electronic cigarette for a defined period of 4.2 seconds. After 12 minutes and 4.2 seconds, the Arduino resets the interval and waits another twelve minutes to send the digital pin output
**Exogenous RNA Spike**

The DNA for the fluorescent protein mClover, a T7 promoter, and a T7 terminator were PCR amplified out of a pBAD-Clover and pET-23a vector via PCR. The primers for this were designed so that the 3’ end of the T7 promoter and 5’ end of Clover overlapped, and that the 3’ end of Clover and the 5’ end of the T7 Terminator overlapped. This allowed for the formation a single product utilizing PCR stitching. After the complete DNA template was stitched, an *in vitro* transcription was carried out using a HiScribe High Yield T7 RNA Synthesis Kit obtained from New England BioLabs (Ipswich, MA). The RNA transcript was confirmed with Urea-PAGE.

**RNA Extraction and cDNA Synthesis**

Qiagen’s RNAProtect (Redwood City, CA) was used in accordance with the manufacturers specifications for solid media. Before lysis, the cell number was normalized at 600 nm using bacterial light scattering properties. The samples were enzymatically and mechanically disrupted using lysozyme, Proteinase K (New England BioLabs, Ipswich, MA), and 0.1 mm zirconia beads (Research Products, Mt. Prospect, IL). The lysates were normalized to the lowest total protein concentration using Bio-Rads Protein Assay Reagent (Hercules, CA) to account for differing lysis efficiencies. The exogenous RNA spike was doped into each sample thereafter. The total RNA was purified using Qiagen’s RNAPure Mini Kit (Redwood City, CA). The total RNA was inspected for quality and purity using Urea-PAGE and UV spectroscopy, and was normalized to the lowest concentration at 260 nm. A cDNA library was created using Bio-Rad’s iScript cDNA Synthesis Kit (Hercules, CA).
**qPCR**

Quantitative PCR was performed with Bio-Rad Sso Fast EvaGreen Supermix in a Bio-Rad CFX96 Real-Time System (Hercules, CA). Gyrase subunit A (*gyrA*) was chosen as an endogenous reference (housekeeping gene). *gyrA* is a topoisomerase responsible for catalyzing negative supercoiling during DNA replication. It alleviates the strain caused by the unwinding action of DNA helicase, and is considered to be an essential gene.\(^{50}\)

The virulence gene primers are listed in Table 2. The data was analyzed using the \(2^{-\Delta Ct}\) method to determine significance between groups normalized to *gyrA*, and the \(2^{-\Delta\Delta Ct}\) method to determine experimental fold change relative to the control. Equations 1, 2, and 3 in the methods section were used to calculate these values. The Ct values were calculated manually in excel from the raw quantification data, where the Cq was arbitrarily set to 500 RFU in the most linear portion of the log2 transform.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein Product</th>
<th>Forward Primer 5' -&gt; 3'</th>
<th>Reverse Primer 5' -&gt; 3'</th>
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<td>SpeB</td>
<td>Streptococcal Pyrogenic Exotoxin B</td>
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<td>AGTCTCTGAACGCTTCTCC</td>
</tr>
<tr>
<td>sda</td>
<td>Streptodornase</td>
<td>GGAGGACATGGATATGCTAAATCA</td>
<td>AAATTTCCGTGTATCAGGGA</td>
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<tr>
<td>ska</td>
<td>Streptokinase</td>
<td>TCTTTTGGATGGTTGCACT</td>
<td>TTGGCTGTGTGGACAGATG</td>
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<tr>
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<td>Streptolysin O</td>
<td>GCTACTGACGGCAGCTCTTA</td>
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<tr>
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<td>Streptolysin S</td>
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<td>GCGTATAACTTCCGGCTACCA</td>
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</table>
Results

Clover RNA Spike

Multiple stitching polymerase chain reactions (lanes 3-6) produced bands at ~950 bp and ~750 bp, despite being amplified from a homogenous gel purified product (Figure 2). The full-length stitch product is 969 base pairs.

This two-product trend continued throughout the RNA transcription procedure. The transcription of the stitch product, as indicated by an arrow (Figure 5), ran between the 1500 bp 16S ribosomal subunit and the 363 bp tmRNA bands, putting it in a reasonable size range (Figure 5). The identity of the amplicon was confirmed after being spiked into a cell lysate, purified on a silica column, converted to cDNA, and amplified via qPCR.

Figure 5: Ethidium bromide gel with the PCR stitch product at 969 base pairs.
After being amplified via qPCR the melt curves of each gene product was examined and compared to the values obtained from Northwestern’s Oligocalc web based software (Figure 4, Table 2). The melt curve of Clover indicates a tm of 87.5 °C, with a small aberration at 75 °C (Figure 4).

**Figure 6**: Urea-PAGE of purified and raw transcription products. The transcription of the stitch product is 879 Bases, and indicated by a black arrow. The positive control Luciferase is 1.8 kbs.

**Figure 7**: Melt curve derivatives of RT-qPCR products
Table 2: Melting temperatures of virulence gene products. An asterisk denotes heterogeneous melt profiles.

<table>
<thead>
<tr>
<th>Gene Product</th>
<th>Product Size (bp)</th>
<th>Tm (°C)</th>
<th>Nearest Neighbor (°C)</th>
<th>Observed Tm (°C)</th>
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</thead>
<tbody>
<tr>
<td>ScpA</td>
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<td>77.67</td>
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<td>79.02</td>
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<td>78.35</td>
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<td>Clover*</td>
<td>108</td>
<td>86.4</td>
<td>85.69</td>
<td>88.5</td>
</tr>
</tbody>
</table>

The RT-qPCR products were run on an ethidium bromide agarose gel to demonstrate consistency between observed and expected product size (Figure 6).

Figure 8: RT-qPCR products on ethidium bromide agarose gel.
**Figure 9:** Total RNA extraction from the experimental and control cell lysates.
**Gene Expression**

The $\Delta$Ct (equation 1) was calculated for both the experimental and control group of each virulence gene product. A student’s t-test (Welch’s correlation) was performed using the $2^{-\Delta\text{Ct}}$ values to determine whether the differences between groups were statistically significant. When *S. pyogenes* was grown in the presence of aerosolized propylene glycol, no significant difference between the experimental and control $2^{-\Delta\text{Ct}}$ values was observed.

**Equation 1:** $\Delta \text{Ct} = [(\text{Ct gene of interest}) - (\text{Ct internal control})]$

![Virulence Factor $\Delta$Ct Values](image)

**Figure 10:** $2^{-\Delta \text{CT}}$ values for treatment and control groups. No significant difference between experimental and control groups was observed using an unpaired Student’s t test assuming a normal distribution. Error bars represent a 95% confidence interval.
The fold change in transcription (equation 2) was calculated for each gene product and displayed in figure. The standard error of measurement considering error propagation was calculated using equation 3, where $s_1$ = standard deviation of $\Delta$Ct experimental and $s_2$ = standard deviation of $\Delta$Ct control. -1 and 1 fold change in transcription indicate no change in transcription occurred.

**Equation 2:** $2^{-\Delta \Delta \text{Ct}} = 2^{-[(\Delta \text{Ct Experimental}) - (\Delta \text{Ct Control})]} = 2^{-[(\text{Ct GOI exp}) - (\text{Ct GOI cont}) - (\text{Ct IC exp}) - (\text{Ct IC cont})]}$

**Equation 3:** $S = \sqrt{(s_1^2) + (s_2^2)}$

**Fold Change in Expression as a Result of Propylene Glycol Exposure**

![Figure 11: Fold change calculated using equation 2. Error bars represent the error propagation using equation 3. Fold changes of -1 and 1 depicts no change in expression.](image)
Discussion

This thesis sought to explore the relationship between virulence gene expression and aerosolized propylene glycol, a primary constituent of electronic cigarette juice. It was hypothesized that the gram-positive pathogen \textit{S. pyogenes} may be able to survive aerosolized propylene glycol treatment when grown on solid media and mount a stress response through virulence pathways.

Though total growth inhibition was observed if freshly inoculated media was placed in the presence of aerosolized propylene glycol, \textit{S. pyogenes} was able to persist if allowed to reach exponential phase before exposure. This provides evidence that GAS has the ability to mediate a stress response only when it has matured and begun biofilm formation on a solid substrate. Because previous studies have only characterized the bactericidal action of PG when bacteria are in aerosolized\textsuperscript{8-10} or in broth\textsuperscript{44} conditions, the results of this study have presented a unique scenario where PG is not 100\% effective in killing gram positive pathogens.

Originally, an RNA spike was to be used for normalization purposes, but issues controlling for lysis and extraction efficiency prompted a change of protocol. Mairhofer’s work describing the run-through transcription process demonstrates that T7 RNA polymerase is prone to transcribing RNA even after reaching the poly U stop sequence, resulting in a longer message than expected.\textsuperscript{52} The variable fidelity of T7 RNA polymerase would explain the two bands observed in figure 5, however, this phenomenon does not explain the banding pattern in figure 4. The heterogeneous nature of the spike as it was used in this study should not have contributed to the RT-
qPCR normalization issues experienced; it should have been equally heterogeneous in both the experimental and control samples. There may be an opportunity to purify the stitch transcription product in the future, allowing for quantification, and thus the ability to determine the molarity. The spike could then be used for absolute expression studies. If this study is to continue, purification the RNA spike or validation of a set of endogenous reference genes should be performed. The geometric mean of the log transformed CT values would serve as the internal reference.\(^4\)

In the future, a more comprehensive survival assay could depict PG mediated growth inhibition with higher resolution. Plates inoculated with a gradient of cell densities could be added to the chamber in 2-hour intervals. After 6-8 hours in the chamber, replica plating could be performed to determine cell viability, the goal being to elucidate the point in time where GAS becomes resistant to PGs bactericidal effects.

Propylene glycol has a potent bactericidal effect on *S. pyogenes* during the colonization phase, but the molecular mechanism remains unknown. Proposing plausible inhibition routes may be worth exploring, particularly whether or not propylene glycol has the ability to coat the cell wall and affect the quorum sensing abilities of GAS. Licheng and colleagues demonstrated that high molecular weight polyethylene glycol (PEG 15-20) has the ability to prevent *P. aeruginosa* adhesion to host tissue, and ultimately inhibits the expression of virulence genes activated by quorum sensing.\(^5\)

Using standard plate counting methods and electron microscopy, it was determined that PEG did not have an effect on *P. aeruginosa* viability, but was acting as a hydrophobic buffer between gut epithelial cells and the pathogen, preventing colonization. It may be
possible that aerosolized PG is coating GAS in early growth phases and preventing it from accessing nutrients necessary for survival, while also eliminating a means of communication and cooperation between colonies.

The periods of incubation where growth inhibition occurs disallows the analysis of gene expression because total RNA is difficult to extract in any appreciable quantity. This initial inhibition stage is the most interesting because GAS is most likely to mount a stress response during this time. The development of a virulence gene reporter construct would allow for expression quantification during this interval, and provide clues as to how regulatory pathways are utilized to overcome chemical antagonists like PG.

A positive control for upregulation would provide an additional lens to view biological significance of the observed gene expression changes effected by propylene glycol. This is accomplished by making blood agar plates with a 1mM dose of potassium ferricyanide. Potassium ferricyanide places immense oxidative stress on microorganisms, affecting the RofA regulon in GAS. The upregulation of RofA results in the increased transcription of fibronectin binding protein, providing a standard for a hyper-virulent phenotype.

This study has reasonably ruled out the possibility that PG mediates changes in virulence gene expression effected by the previously characterized stand-alone and two component regulatory systems. Despite this, the study was able to establish a consistent means to test the effects of aerosols on cells and narrow down the mechanism behind PGs bactericidal nature. This study addressed only the effects of
carrier component of electronic cigarette juice; the obvious follow-up experiment would be measuring gene expression as a response to commercial electronic cigarette juice.
Appendix A: IBC Approval Letter

University of Central Florida

Biological Agent Registration Form-IBC Use Only

IBC Committee Use Only

Registration # 15-14

Review Date: 10/28/2015 Approval Date: 11/03/2015 Expiration Date: 11/03/2018

Application reviewed by: [ ] Full Committee [ ] BSO

[ ] Approved [ ] Modifications required for approval

[ ] Denied

Biosafety Level Required: [ ] Exempt [ ] BSL-1 [ ] ABSL-1
[ ] BSL-2 [ ] ABSL-2
[ ] BSL-3 [ ] ABSL-3

Biosafety Cabinet Required:
If yes, is the BSC currently certified:
Biosafety lab audit has been completed by EH&S:

[ ] Yes [ ] No

Note: The biosafety audit must indicate that all lab personnel are up to date on training with EH&S, and that appropriate waste containers and PPE for the listed biohazards are present in the lab. If not, the PI must indicate when these requirements will be added/completed.

Lab personnel are up to date on training EH&S:

[ ] Yes [ ] No

Appropriate waste containers and PPE present in lab:

[ ] Yes [ ] No

PI has conducted risk assessment and proposed standard operating procedures (SOPs) including decontamination/spill clean-up, and waste disposal methods:

[ ] Yes [ ] No

Committee Notes:
No rDNA work associated with this project.
RNA will be extracted from S. pyogenes for mRNA analysis.

IBC Chair Signature: Karl X. Chai Date: 11-3-15

BSO Signature: Sabena Blakeney Date: 11/03/2015
Bibliography


43. Rouadi P. NR. A technique to measure the ability of the human nose to warm and humidify air.