Chapter 4

Effects of Electronic (e)-Cigarette Vapor on Staphylococcal Virulence: Are E-Cigarettes Safer than Conventional Cigarettes?

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Abstract

In recent years, electronic (e)-cigarettes have dramatically increased in popularity as an alternative to conventional cigarettes. Little is known about the effects of e-cigarette vapor (EV) on bacteria that colonize the nasopharynx, including methicillin-resistant *Staphylococcus aureus* (MRSA). As most cases of pneumonia can be traced to bacteria in a patient's nasopharynx, increased virulence in potential pathogens could have direct consequences clinically for these patients. And because bacterial colonizers are spread between humans, increased virulence in one subject has implications for the community. There is accumulating evidence that exposure to cigarette smoke (CS) increases the pathogenicity of MRSA, as well as its dampening effects on the host immune system. EV exposure has also been demonstrated to increase MRSA virulence both *in vitro* and in a murine model of pneumonia. In this chapter, we will compare the virulence changes reported in MRSA exposed to CS vs. those exposed to EV, as well as proposed mechanisms and therapeutic targets.

Keywords: electronic cigarettes, e-cigarette vapor, cigarette smoke, staphylococcal virulence, MRSA, pneumonia

1. Introduction

Smoking is a leading cause of preventable morbidity and mortality worldwide, with an estimated 6 million premature deaths per year (10% of all deaths globally) attributable to cigarette smoke (CS) exposure [1]. Health ramifications of smoking include cardiovascular

disease, malignancy, and chronic obstructive pulmonary disease [1]. In addition, smokers have increased rates of serious infections of the respiratory tract.

Tobacco smoke has many known carcinogenic and inflammatory effects on the airways and lungs [2]. Each puff of a cigarette exposes the epithelial and immune cells of the airway to more than 7000 chemicals including toxins such as acrolein, formaldehyde, and benzo*(a)*pyrene, as well as nicotine, the compound responsible for the addictive potential of both conventional cigarettes and e-cigarettes [1]. In chronic smokers, this chemical bath induces an inflammatory response in the airway that simultaneously damages the structural integrity of the airways and reduces the ability of host cells to clear pathogens from the airway [1–3]. Bacterial colonizers of the airways, including pathogens such as methicillin-resistant *Staphylococcus aureus* (MRSA), are exposed to the same toxic cigarette smoke milieu.

A newly fashioned nicotine delivery device, known commercially as electronic (e)-cigarette or vape pen was introduced to the international market for the first time in 2007 [4]. E-cigarettes have experienced a dramatic surge in popularity in the last decade. Their use among youth is a pressing public health issue, with prevalence of use surpassing that of tobacco cigarettes [5]. About 13.4% of USA youth are current users of e-cigarettes, a higher rate than that of traditional tobacco use. This corresponds to 3 million middle and high school students, up from 2.46 million in 2014 [6]. Moreover, e-cigarettes were found to increase smoking initiation by more than 200% with a prediction to achieve a corresponding 6% increase in smoking prevalence by 2060 [7]. One out of every four current e-cigarettes users in Spain have never smoked before [7, 8].

E-cigarettes consist of a cartridge containing nicotine in a propylene glycol (PG) and/or vegetable glycerin (VG) solvent that is heated and then vaporized via an atomizer; this vapor is subsequently inhaled, resulting in similar nicotine delivery to the bloodstream as conventional cigarettes. E-cigarettes have been promoted as a safer alternative to tobacco products, with fewer adverse effects and as being efficient in smoking cessation. In this respect, a recent study conducted in 28 European countries suggested a positive public health impact for e-cigarette for the tobacco smokers accompanied by minimal potential harm due to use by never smokers [9]. However, this study based its findings on a little-harm model of e-cigarettes, which is not supported by scientific data. E-cigarette components have been shown to contain many hazards for the respiratory system. In 2009, the US FDA found detectable levels of toxic cancer-causing chemicals in e-cigarette cartridges [10]. Many other components such as aldehydes, metals, volatile organic compounds, phenolic compounds, polycyclic aromatic hydrocarbons, and tobacco alkaloids were identified in e-cigarette cartridges, refill solutions, and aerosols [11]. All of these hazardous components in turn will come in contact with respiratory colonizing pathogens, including *S. aureus,* and may promote pro-virulent changes.

S. aureus are Gram-positive cocci with the potential to cause serious infections including bacteremia, endocarditis, and pneumonia, as well as skin and soft tissue infections ranging from impetigo to necrotizing fasciitis. More than one-third of the general population is asymptomatically colonized with *S. aureus* on either a transient or persistent basis, most frequently in the nose, throat, and perineum [12–14]. These colonizers are frequently the source of host staphylococcal infections, and patients with positive nasal swabs for *S. aureus* are known to

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be at high risk of nosocomial and surgical site infections [15]. Persistent carriers frequently have a higher bacterial load and a corresponding increased risk of subsequent staphylococcal infection relative to intermittent carriers [15].

Smokers are more likely than nonsmokers to be colonized with *S. aureus*, defined as >500 colony forming units (CFU) detected, with rates of colonization increasing with duration and frequency of smoking [14, 16, 17]. Smokers have an increased incidence of invasive staphylococcal infections than nonsmokers and have higher mortality as well [17–19]. Additionally, smokers are at greater risk of being colonized with antibiotic resistance *S. aureus* (MRSA) than nonsmokers. This finding is of note because MRSA infections have higher morbidity and mortality than methicillin-sensitive strains [20, 21]. Accumulating evidence suggests that this susceptibility to MRSA/staphylococcal infection may reflect not only the impairment of host defenses induced by chronic exposure to cigarette smoke, but also provirulent changes in commensal MRSA itself as a consequence of the same chronic cigarette smoke exposure.

To our knowledge, there are no epidemiologic studies of staphylococcal infection in e-cigarette users. However, recent work from our laboratory suggests that e-cigarette vapor (EV) may induce a similar stress response as conventional cigarette smoke in exposed *Staphylococcus*, resulting in a propathogenic phenotype.

2. Conventional cigarette smoke promotes increased staphylococcal virulence

MRSA often colonizes the nasopharynx, where it is exposed to everything that an individual inhales, including cigarette smoke (CS) or e-cigarette vapor (EV). Bacteria from the nasopharynx have the potential to travel to the lungs, causing pneumonia, or to the skin, where compromises in the epithelial barrier such as ulcers, abrasions, or surgical incisions provide ports of entry for invasive staphylococcal infection. The initial stage preceding infection is colonization of the human host. Transient colonization of the nasopharynx by *S. aureus* is more common in healthy individuals than persistent colonization (30 vs. 20%), which usually entails a larger bacterial burden and increased risk of infection [15]. Smokers are known to have higher rates of persistent colonization of the nasopharynx by MRSA relative to the general population [17]. The phenotypic changes in *S. aureus* induced by *in vitro* CS exposure favor persistent colonization via increasing adhesion to host cells and by inducing biofilm formation, reducing the ability of host immune effectors to limit colonization [22].

2.1. MRSA hydrophobicity changes induced by cigarette smoke

Our own studies demonstrate that CS extract (CSE) exposure results in a dose-dependent increase in MRSA surface hydrophobicity that is persistent and possibly even heritable [23, 24]. Increased hydrophobicity is associated with increased bacterial interactions with host epithelial cells [25]. Thus, we evaluated the adherence of MRSA to human keratinocytes (HaCaT cells) *in vitro* and found that pretreatment with CSE increased adherence from 28% (control) to 52% (CSE-MRSA). These findings are likely due to increased interaction with

epithelial cell surfaces because of the increased surface hydrophobicity induced by CSE exposure. Increased capacity to adhere to epithelial cells as a consequence of CS exposure may explain the increased MRSA carriage rates in smokers.

2.2. Staphylococcal surface charge changes induced by cigarette smoke

CSE exposure induced dramatic changes in surface charge, in a dose-dependent manner. With increasing concentrations of CSE, the surfaces of *S. aureus* and MRSA became less negative. The negative surface charge of bacteria is protective against a variety of host defense mechanisms, including antimicrobial peptide (AMP) binding. Changes in surface charge were persistent for over 24 h post-CSE exposure. These changes suggest that the CSE exposure may result in persistent, heritable changes. Additionally, daily intermittent exposure to CSE over the course of 3 or 4 days—reflecting the pattern of exposure experienced by the nasopharyngeal flora of a smoker—resulted in an additive increase in surface charge alterations.

Cigarette smoke contains thousands of compounds, making the identification of specific etiologic agent inducing change in surface charge difficult. However, we hypothesized that reactive species such as free radicals and carbon monoxide likely played a role. To assess the contribution of reactive species, CSE was stored for 24 h at 4°C to allow time for volatile gases to evaporate and for some degradation of reactive oxygen species. The "aged" CSE induced a less-anionic surface charge in exposed MRSA; however, it had significantly less potency relative to fresh CSE. Thus, while reactive species account for some measure of the observed surface changes in MRSA, some of the more stable elements of CS contribute as well. One such component, present in both fresh and aged CSE—as well as e-cigarette vapor—is nicotine. We found that nicotine at 3 and 6 mg/mL induced a dose-dependent shift toward a less negative surface charge, suggesting that it likely plays a role in the altered surface charge of CSE-exposed MRSA. Finally, we sedimented CSE and exposed MRSA solely to these particulates and found no effect on surface charge.

The cell surface charge changes are mediated in part by expression of the *mprF* gene, which encodes a membrane protein involved in the shift to a less negative surface charge. Exposure to CSE induced a 1.8-fold increase in expression of *mprF* RNA, and CSE did not induce significant surface charge change in an *mprF* knock-out strain of *S. aureus* (SA113 Δ*mprF*), relative to wild-type bacteria, further supporting an important role of this pathway in this aspect of the staphylococcal response to CS exposure.

2.3. Cigarette smoke exposure increases *S. aureus* resistance to antimicrobial defenses

Aspiration of nasopharyngeal colonizers is a common occurrence and provides an opportunity for potential pathogens to cause airway infection. Numerous immunologic defenses are in place to protect against this outcome, including mechanical clearance of bacteria by the mucociliary elevator and destruction of remaining bacteria by immune cells within the airways. Alveolar macrophages, which destroy bacteria by mechanisms including phagocytosis, antimicrobial peptide (AMP) production, and respiratory burst, are an important component of host defense against pulmonary infection by bacteria.

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CSE exposure may induce MRSA resistance to the bacteriocidal activities of these cells by multiple mechanisms. *In vitro*, CSE-exposed MRSA was resistant to killing by a murine alveolar macrophage cell line (MH-S), with a fourfold increase in survival relative to control ($p < 0.0001$). This outcome was not due to a cytotoxic effect on the macrophages, as macrophage cell death rate was comparable regardless of whether they were infected with CSE-treated MRSA or control bacteria. Phagocytosis of fluorescently tagged MRSA-GFP was unaffected by CSE exposure—suggesting that the difference in survival reflected that the CSE-exposed MRSA were better able to endure intracellular killing mechanisms including exposure to antimicrobial peptides and other toxic compounds and reactive oxygen species found in the phagolysosome. Indeed, CSE exposure increased resistance to killing by the human AMP LL-37, with twofold increases in both MIC and MBC relative to control bacteria. Decreased susceptibility to AMPs, which are produced by neutrophils, macrophages, and epithelial cells, suggests a significant increase in pathogenicity.

Potential mechanisms of this resistance include changes in cell surface charge and decreased rate of cell division induced by CS. Rapidly dividing bacteria are more sensitive to many antimicrobials, including conventional antibiotics and AMPs. Kristian et al. showed that growth suppression using bacteriostatic antibiotics allowed *S. aureus* decreased susceptibility to killing by AMPs [26]. Growth curves at various concentrations of CSE-demonstrated inhibition of bacterial growth in a CSE dose-dependent fashion. The growth defect induced by CSE resolved with removal of CSE; however, it is possible that a clonal population continues to divide slowly and that this population is better equipped to persist in the face of subsequent stressors.

Interestingly, the changes induced by CSE exposure also seemed to induce resistance to membrane solublization by detergent. CSE-MRSA had similar death rates to control bacteria during the first few minutes of incubation with detergent (Triton-X), but demonstrated improved survival subsequently, suggesting further protective cell membrane changes induced by CSE.

Findings *in vivo* in a murine model of pneumonia supported *in vitro* results, with CSE exposure resulting in increased staphylococcal persistence in the lungs and an increase in overwhelming infection. Mice infected with CSE-exposed MRSA had increased bacterial burdens in the lungs at 8 and 24 h relative to those infected with control bacteria, demonstrating that CSE-MRSA are better able to persist in the face of pulmonary immune defenses. Additionally, 40% of mice infected with a higher inoculum of CSE-exposed MRSA died within 48 h, vs. only 10% of those infected with unexposed MRSA.

2.4. Potential consequences of virulence changes induced by cigarette smoke

While pulmonary infection with MRSA is devastating, much of the morbidity and mortality associated with MRSA infections is due to soft tissue infections. Because bacteria colonizing the nasopharynx do not necessarily remain there, propathogenic changes induced by CS in the nasopharynx are of concern for staphylococcal infections beyond the pulmonary system. Coughing, sneezing, etc. lead to significant opportunity to transfer MRSA from nasal passages and upper airway to the skin, where an increased capacity to adhere to and invade epithelial cells—as well as resistance to AMPs—reflect serious potential consequences of the

pro-pathogenic effects of cigarette smoke for skin and soft tissue infections. Finally, MRSA are transmitted between humans in the community as well. Thus, more virulent strains generated by CS exposure may put the community at higher risk of invasive staphylococcal diseases.

3. E-cigarette vapor increases staphylococcal virulence and impairs innate immune function

As the popularity of e-cigarettes skyrockets, it becomes increasingly important to establish whether e-cigarettes are indeed a safer alternative to conventional cigarettes. Simply, it is a battery operated device designed to deliver nicotine with flavors and other chemicals to users in vapor form instead of smoke. E-cigarette vapor (EV) contains a far more limited array of chemicals than cigarette smoke, resulting from vaporization of e-liquid containing nicotine, propylene glycol (PG), and vegetable glycerin (VG) solvents, with different flavors added in some cases. While PG is not harmful when ingested through the GI tract and is generally recognized as safe for use as a food additive [27], high wattage heating of these solvents results in the production of formaldehyde, aldehyde, and acrolein, resulting in significantly increased exposure of the airways to formaldehyde relative to a pack-day smoker [28]. The absorption of nicotine in blood showed no difference between the latest generation e-cigarettes and conventional cigarettes. Nicotine itself is toxic at high doses, and as described in the preceding section, induces a shift in MRSA toward a less negative surface charge which confers resistance to AMPs. Thus, e-cigarette "vaping" is exposing the colonizing bacteria to at least two components (formaldehyde and nicotine) with the potential to induce a significant stress response.

3.1. MRSA growth suppression by e-cigarette vapor

We tested the growth kinetics of MRSA cultures during exposure to different components of EV extract (EVE) including nicotine, PG, and VG. We also tested a number of different brands of e-cigarettes. EVE suppressed MRSA growth, with cultures failing to achieve logarithmic phase. While nicotine mildly inhibited MRSA growth in a dose-dependent fashion, the vaporized vehicles likely account for most of the suppressive effects observed. Vaporized PG and/or VG nearly abrogated MRSA growth, to the same degree as EVE [29]. Interestingly, MRSA growth suppression was observed in four of five brands, suggesting that the etiologic agent is a common component across brands. Thus, as with cigarette smoke, EV imposes significant stress on Staphylococcal cells. Bacterial survivors of the noxious exposure are likely to be hardier, and thus more difficult to kill.

3.2. Increased hydrophobicity, adherence, and invasion of keratinocytes by e-cigarette vapor exposed MRSA

As with conventional CS, EV promotes a shift in phenotype that supports persistent MRSA colonization of the epithelium, as well as increasing the risk of invasive infection. Hydrophobicity was markedly increased following EV exposure (by 31%), with a corresponding doubling of

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MRSA adherence to the human keratinocyte HaCaT cell line. EVE-exposed MRSA demonstrated increased invasion of and intracellular persistence within HaCaT cells. These changes do not appear to be induced by nicotine, as increasing the nicotine content by fivefold had no effect on adherence and invasion.

3.3. E-cigarette vapor increases MRSA resistance to human antimicrobial peptide LL-37

Increased survival of internalized MRSA suggests that e-cigarette exposure may induce resistance to killing by epithelial cells. This effect is at least in part a consequence of moderately increased resistance to killing by antimicrobial peptides (AMPs). One of the established mechanisms for bacterial virulence is alterations in surface charge. Most bacteria have predominantly anionic surfaces, which are targeted by the human innate immune system [30, 31]. One of the human AMPs, LL-37, acts through charge interactions between its cationic surface and the bacterial lipids which are negatively charged [30–32]. EVE exposure increased the MIC of LL-37 from 7 uM in unexposed MRSA to 10 uM in EVE-MRSA ($P = 0.014$). As with conventional CS, EVE exposure induces a shift in MRSA toward a less negative surface charge. In turn, this reduces the propensity of the cationic LL-37 to bind the bacterial surface as it must for antimicrobial activity. This transition in surface charge was independent of the level of nicotine in EVE.

3.4. MRSA biofilm induction by e-cigarette vapor

EV has other pro-virulent effects on MRSA that may allow the bacteria to cause more severe infections—it promotes a moderate increase in biofilm formation. Antibiotics and our own immune cells have difficulty penetrating biofilm to kill embedded bacteria, thus allowing colonization (or infection) to perpetuate. The increased predilection for biofilm following EV exposure may reflect a response to nicotine in the vapor, as nicotine alone induced dose-dependent increases in biofilm formation. Interestingly, the increased biofilm formation occurring in response to conventional CS seemed to be due primarily to oxidative stress [29]. In our studies, MRSA incubated with only nicotine produced more biofilm than control. In the exposure to either EV or CS, a novel pathway in inducing MRSA biofilm was suggested [23, 29].

3.5. E-cigarette vapor increases MRSA virulence in a mouse model of pneumonia

In vivo studies in mice also suggested that EV promotes propathogenic features. The same murine pneumonia model used to evaluate CS effects on MRSA was employed to determine EV effects. Mortality was increased in mice infected with EV-MRSA (25 vs. 0% in mice infected with control MRSA, $p < 0.05$). In addition, bacterial burdens were 10-fold higher in mice infected with EV-MRSA relative to mice infected with MRSA controls. Exposure to EV enhanced both MRSA virulence and MRSA survival [29].

3.6. E-cigarette vapor induction of MRSA virulence gene expression

Similarly to the effect of CS exposure on MRSA virulence, MRSA exposed to EV was more virulent than controls in the biological model. Thus, the expression of several well-known

virulence factors after EV exposure was evaluated. Panton-Valentine leukocidin (*pvl*), α-hemolysin (*hla*), coagulase (*coa*), α-phenol soluble modulin (*psm-α*), intracellular adhesion (*icaA*), staphylococcal protein A (*spa*), and quorum sensing (*agrA*) were quantified relative to 16s rRNA as a housekeeping gene. After EV exposure, the expression of *coa* and *pvl* increased by 1.68- and 1.56-fold, respectively [29]. Expression of *spa* did not change with EV exposure while *icaA, agrA, hla,* and *psm* decreased.

4. Cigarette smoke versus e-cigarette vapor

These data provide suggestive evidence that EV induces pro-virulent changes in MRSA comparable in type and magnitude to those promoted by exposure to conventional CS. Both EV and CS exposures resulted in shifts toward more hydrophobic and less anionic surface charges, with increased adherence and invasion of epithelial cells, and resistance to killing by the AMP LL-37. Additionally, the phenotypic changes induced by exposure to both inhalants result in significantly increased virulence in a mouse pneumonia model. Without running assays in parallel, however, definitive comparisons cannot be made (**Table 1**).

Both CS and EV exposure result in a similar profile based on these assays, likely reflecting a general stress response. However, the primary component of the inhalants inducing these

	Cigarette smoke		**E-cigarette vapor**	
Growth	↓↓	***	↓↓	****
Hydrophobicity	↑↑	****	↑	*
Adherence	↑	*	↑↑	**
Invasion	↑↑	**	↑↑	*
Surface charge changes	++++	****	+	****
Resistance to killing by:				
Macrophage	↑↑↑↑	****		
Antimicrobial peptide (AMP)	↑↑	*	↑	*
Cell lysis	↓	***		
Biofilm formation			↑↑	**
Virulence *in vivo*	↑↑	*	↑	*

*$p < 0.05$.
**$p < 0.01$.
***$p < 0.001$.
****$p < 0.0001$.

Table 1. Comparison of the effects of cigarette smoke and e-cigarette vapor on MRSA pathogenicity factors and virulence.

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phenotypic changes may be different. This likely reflects that CS contains a multitude of toxic chemicals, with a more limited range found in EV. However, both nicotine and degradation products of the e-cigarette liquid solvents are clearly sufficient to induce concerning changes in MRSA *in vitro*. The effects of both CS and EV on MRSA in a physiologic setting may be synergistically negative due to the interplay of increased virulence with immunosuppressive effects of these substances on airway cells. Current studies are limited to animal models, and more research must be carried out to evaluate whether these changes reflect those observed following physiologic exposure in human users of both conventional and e-cigarettes. Additional avenues of research include evaluating the effects of exposure to these substances on staphylococcal soft tissue infections and bacteremia. In addition, epidemiologic studies on the incidence of staphylococcal infections in e-cigarette users are needed to assess the likelihood of our findings having a physiologic correlate.

Comparison of the effects of cigarette smoke and e-cigarette vapor on MRSA pathogenicity factors and virulence.

5. Possible downstream consequences of CS and EV on antibiotic resistance in MRSA

The effects of EV and CS on MRSA hydrophobicity, surface charge, virulence, and resistance to killing by the innate immune system host defense antimicrobial peptides have potentially harmful implications on antistaphylococcal therapy. Vancomycin and daptomycin are the only drugs approved by the US FDA for the treatment of MRSA bacteremia and endocarditis [33]. When MRSA causes endocarditis, it is due to the patient's colonizing strain. MRSA strains that are induced to relative resistance to cationic host defense peptides, such as platelet microbicidal proteins (PMPs), are associated with a proclivity to lead to persistent bacteremia, endocarditis, and metastatic infectious foci [34]. In other words, durable changes in MRSA physiology by EV and CS may have implications on the ability of this pathogen to establish endovascular infection in cases of an otherwise transient bacteremia that is thought to occur periodically in healthy hosts but otherwise rapidly quenched by the innate immune system. Furthermore, cross-resistance between host PMPs and cathelicidin and vancomycin and daptomycin have been well-documented [34–36]. Thus, EV and CS may have serious implications on the proclivity of MRSA to develop heteroresistance to these drugs, even before the agents are administered to the patient.

Compounding this, resistance concern is the fact that persistent lingering of MRSA *in vivo*, as a result of resistance to host defense peptide killing, further creates a selective pressure environment conducive to further cationic peptide resistance, and hence resistance to antimicrobial therapy [37]. These extrapolative hypotheses of the effects of EV and CS have yet to be proven, but if they are, have far greater public health implications than once thought.

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