Identification of a marker of infection in the breath using a porcine pneumonia model

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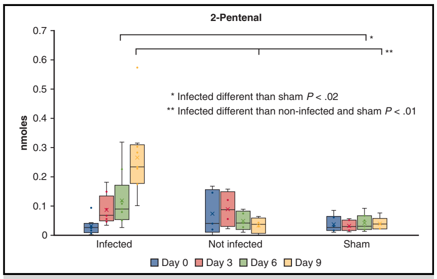
ABSTRACT

Objective: Pneumonia, both in the community and the hospital setting, represents a significant cause of morbidity and mortality in the cardiothoracic patient population. Diagnosis of pneumonia can be masked by other disease processes and is often diagnosed after the patient is already experiencing the disease. A noninvasive, sensitive test for pneumonia could decrease hospitalizations and length of stay for patients. We have developed a porcine model of pneumonia and evaluated the exhaled breath of infected pigs for biomarkers of infection.

Methods: Anesthetized 60-kg adult pigs were intubated, and a bronchoscope was used to instill a solution containing 12×10^8 cfu of methicillin-sensitive *Staphylococcus aureus* or a control solution without bacteria (Sham) into the distal airways. The pigs were then reintubated on postoperative days 3, 6, and 9, with bronchoscopic bronchial lavages taken at each time point. At each time point, a 500-mL breath was captured from each pig. The breath was evacuated over a silicon microchip, with the volatile carbonyl compounds from the breath captured via oximation reaction, and the results of this capture were analyzed by ultra-high performance liquid chromatography mass spectrometry.

Results: A total of 64% of the pigs inoculated with methicillin-sensitive *S. aureus* demonstrated consolidation on chest radiography and increasing counts of methicillin-sensitive *S. aureus* in the bronchial lavages over the span of the experiment, consistent with development of pneumonia. Analysis of the exhaled breath demonstrated 1 carbonyl compound (2-pentenal) that increased 10-fold over the span of the experiment, from an average of 0.0294 nmol/L before infection to an average of 0.3836 nmol/L on postoperative day 9. The amount of 2-pentenal present was greater in the breath of infected pigs than in the noninfected pigs or the sham inoculated pigs at postoperative days 6 and 9. Using an elevated concentration of 2-pentenal as a marker of infection yielded a sensitivity of 88% and specificity of 92% at postoperative day 6, and a sensitivity and specificity of 100% at postoperative day 9.

Conclusions: We were able to successfully develop a clinical pneumonia in adult 60-kg pigs. The concentration of 2-pentenal correlated with the presence of pneumonia, demonstrating the potential for this compound to function as a biomarker for methicillin-sensitive *S. aureus* infection in pigs. (JTCVS Open 2023;16:1063-9)



Elevation in 2-pentenal in the breath of pigs developing *Staphylococcus* pneumonia.

CENTRAL MESSAGE

An exhaled carbonyl compound (2-pentenal) serves as a detectable biomarker for pneumonia in a porcine model of *Staphylococcus aureus* infection.

PERSPECTIVE

Ventilator-associated pneumonia remains a significant cause of morbidity and mortality. Identification of biomarkers that might allow for earlier detection of infection could improve patient outcomes. We have identified, in a porcine model of *Staphylococcus aureus* pneumonia, an exhaled biomarker that could allow for preclinical detection of infection.

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Abbreviations and Acronyms

ATM	= 2-(aminooxy)ethyl-*N,N,N*-trimethylammonium
BAL	= bronchoalveolar lavage
MSSA	= methicillin-sensitive *Staphylococcus aureus*
POD	= postoperative day
UHPLC-MS	= ultra-high performance liquid chromatography mass spectrometry

Pneumonia is common disease resulting from an acute lower respiratory tract infection that affects the pulmonary parenchyma in 1 or both lungs. According to the Centers for Disease Control National Center for Health Statistics, pneumonia was responsible for 1.5 million visits to the emergency departments and 41,309 deaths in 2020.[1] In both the community and hospital setting, pneumonia represents a significant cause of morbidity and mortality in the cardiothoracic patient population. Recent studies have demonstrated a prevalence rate for postoperative pneumonia after cardiac surgery between 2% and 10%.[2-4] Postoperative and ventilator-associated pneumonia remains a common cause of intensive care unit and overall hospital morbidity and mortality despite advances being made in the management of this condition.[2] Diagnosis of pneumonia can be masked by other disease processes and is often diagnosed after the patient is already experiencing the disease. The typical diagnostic criteria for diagnosing pneumonia are a fever, increase in the patient's white blood cell count, and infiltrates on chest radiography, although studies have shown up to 35% of cases diagnosed as pneumonia had a negative chest radiograph.[5] Bronchoalveolar lavage (BAL) cultures are the definitive way of diagnosing pneumonia, although that is sometimes impractical clinically. It has been consistently shown in the literature that delay in starting appropriate and adequately dosed antibiotic therapy increased the morbidity and mortality rates, which highlights the importance of early diagnosis of pulmonary infection.[6,7]

During recent years, several studies have investigated using breath gas analysis for noninvasive detection of various diseases.[8] Our group has developed a technique for collecting and concentrating volatile carbonyl compounds from breath,[9] and we have previously demonstrated the detection of specific biomarkers of cancer present in the breath of patients with lung cancer.[10-12] As described previously, "breathomics" refers to the analysis of volatile compounds in exhaled breath that are produced from and are impacted by various metabolic processes occurring within the host.[13] Therefore, changes within concentrations of volatile compounds within a host's exhaled breath may represent changes in metabolic processes occurring within the host as a direct result of microbial infection or as a result of the host's response to inflammation resulting from microbial infection.[14] The analysis of exhaled breath is a promising noninvasive tool for the diagnosis of pneumonia, but its clinical relevance has yet to be established, as several studies in humans have yielded mixed results.[15]

Prior studies have demonstrated the use of porcine models to mimic clinical conditions of severe pneumococcal pneumonia in mechanically ventilated patients; however, the use and analysis of exhaled breath of infected pigs to assess for changes in volatile carbonyl compounds in response to infection have yet to be investigated.[16] After developing a porcine model of pneumonia, we hypothesized that we could identify biomarkers within exhaled breath that could detect the presence of pulmonary infection.

MATERIALS AND METHODS

Induction of Anesthesia and Mechanical Ventilation

The presented animal research was conducted following a protocol reviewed and approved by the Institutional Animal Care and Use Committee of the University of Louisville. Female Yorkshire pigs (50-70 kg) were used for this protocol. For anesthesia induction without having an intravenous catheter in place, intramuscular midazolam was used for premedication if the animal was anxious or easily excitable. This was followed by subcutaneous/intramuscular ketamine/medetomidine. For anesthesia induction in animals with an intravenous catheter in place, propofol alone or in combination with ketamine or ketamine midazolam was used for a smoother and less stressful induction. The choice of premedication and induction medication was made by the veterinarian and dependent on the health status of the animal at the time of induction and which combination would provide the best response in each animal. Pigs were then orotracheally intubated with a 9.0-mm internal diameter and 12.1-mm outer diameter endotracheal tube comprising a high-volume low-pressure cuff (Covetrus). Animals were connected to a Penlon mechanical ventilator. The pig's sedation was then maintained on isoflurane gas with boluses of 50 mg of intravenous sodium thiopental administered as needed to optimize sedation. A propofol/fentanyl continuous infusion was used for general anesthesia if the isoflurane was unable to maintain a surgical plane of anesthesia due to primary lung pathology.

Baseline Data Collection

After successful intubation and sedation was achieved, pigs were transferred to the operating table to perform baseline data collection. Baseline anterior-posterior and lateral chest radiographs were obtained to evaluate for preexisting infiltrates or consolidations within lung fields. A baseline breath sample was collected into a 1-liter Tedlar bag (as described in "Collection of Breath Samples"). A flexible bronchoscope was then introduced into the airway under video guidance and used to obtain BAL samples using 20 mL normal saline solution to confirm absence of preexisting pulmonary infection at baseline.

Collection of Breath Samples

After intubation and adequate sedation, a 3-way valve connector was attached to the endotracheal tube to facilitate collection of exhaled breath. After delivery of normal tidal volume breath to the pig from the ventilator, the endotracheal tube was closed off to the ventilator and opened to the attached 1-liter Tedlar bag (Sigma-Aldrich) to collect breath from a single exhalation (~500 mL).

Preparation of Bronchial Lavage Sample Culture

A baseline BAL sample was submitted on the day of the first procedure. The baseline BAL was plated on sheep blood agar and mannitol salt agar to determine whether or not the pig has any normal flora or baseline underlying infection. Colony growth was reported at 48 hours. Subsequent BAL samples were submitted at postinoculation day 3, day 6, and day 9 to be plated on sheep blood agar and mannitol salt agar to determine any growth of *Staphylococcus aureus.* Growth was reported at 48 hours.

Preparation of *S. aureus* and Sham Inoculum

S. aureus American Type Culture Connection #12600 (methicillin-sensitive *S. aureus* [MSSA]) was grown on sheep's blood agar approximately 24 hours before preparation of the inoculum. Two heavy 4.0 McFarland solutions of the *S. aureus* were prepared in 40 mL each 0.9% sterile saline to be inoculated into the lungs of the study pig. Two vials of 40 mL each 0.9% sterile saline solutions without MSSA component were prepared in a similar fashion to be instilled into the lungs of the Sham control pig.

Inoculation with *S. aureus* (Methicillin-Sensitive *S. aureus*) or Normal Saline Solution (Sham)

After successful intubation and adequate sedation, we performed collection of baseline data (breath collection, AP/lateral chest radiographs, and baseline BAL samples). Each pig was then turned in lateral decubitus position, and the bronchoscope was introduced into the dependent lung to instill a 40-mL solution containing 12×10^8 cfu of MSSA or normal sterile saline (Sham) into the distal airway. The pig remained in this position for 30 minutes before being turned to the lateral decubitus position on the opposite side and repeating the instillation process for the opposite lung. Likewise, the pig remained in this position for 30 minutes after inoculation before being weaned from sedation and extubated. The pig was then transferred to the postoperative area and monitored closely under the care of veterinary staff.

Postoperative Monitoring Provided by Veterinary Staff

After the procedures, each pig was monitored by veterinary staff. Vital signs, activity level, and appetite were monitored to evaluate for clinical signs of developing infection. Pigs were treated symptomatically with supplemental oxygen, antipyretic medications, antinausea medications, and analgesic medication administered as recommended by the veterinary staff to keep each pig comfortable. Antibiotics were not given to treat respiratory infection.

Subsequent Data Collection

Each pig was brought back to the procedure room and reintubated (as described above) on postoperative day (POD) 3, 6, and 9. At each time point, a breath sample and BAL samples from each lung were collected as described in "Collection of Breath Samples" and "Baseline Data Collection." The breath sample was evacuated over a silicon microchip, with the volatile carbonyl compounds from the breath captured via oximation reaction, and the results of this capture were analyzed by ultra-high performance liquid chromatography mass spectrometry (UHPLC-MS). The BAL samples were grown on mannitol salt agar and sheep blood agar (as described in "Preparation of Bronchial Lavage Sample Culture") with growth reported at 48 hours.

Silicon Microreactor

The microreactor chips were fabricated from 4-inch silicon wafers using previously published microelectromechanical systems fabrication techniques.[9,17] The size of the silicon chip is similar to the size of a dime and consists of an array of thousands of micropillars in the microfluidic channel to uniformly distribute gas flowing through the channel. A quaternary ammonium compound, 2-(aminooxy)ethyl-*N,N,N*-trimethylammonium (ATM) triflate, was used to coat the surfaces of the micropillars as previously described.[9,17,18] ATM adsorbs to the silicon dioxide surfaces of the micropillars via electrostatic and hydrogen bond interactions. ATM chemoselectively traps carbonyl compounds in exhaled breath by means of oximation reactions.

Collection of Carbonyl Compounds in Exhaled Breath

The procedure for the capture of carbonyl compounds in air and exhaled breath has been described previously.[9,17,18] To summarize, the exhaled breath collected in 1-L Tedlar bags was drawn through the microreactor chip by applying a vacuum at a flow rate of 7 mL/min. After this process, ATM adducts in the microreactors were eluted with 200 μL of water from a slightly pressurized small vial. More than 90% of ATM adducts were recovered from the microreactors. The eluted solution was analyzed directly by UHPLC-MS, and 5×10^{-9} mol of ATM-acetone-d6 adduct was added to the eluted solution as an internal reference. The concentrations of all carbonyl compounds in exhaled breath were determined by comparison of the relative abundance with that of added ATM–acetone-d6.

Ultra-High Performance Liquid Chromatography Mass Spectrometry

A Thermo Scientific UHPLC-MS system equipped with an automatic sampler, a Vanquish UHPLC, and a Q Exactive Focus Orbitrap Mass Spectrometer was used for the analysis. The UHPLC had an ACQUITY BEH phenyl column (2.1 $\times$ 100 mm, 1.7 μm) for the separation of ATM-carbonyl adducts. The liquid flow rate through the column was set to 0.2 mL/min. The column temperature was stabilized at 30°C. The autosampler tray temperature was set at 8°C, and 5 μL of the sample was injected into the column. Mobile phase A was 0.1% formic acid in water, and mobile phase B was acetonitrile. The instrument was operated in the positive electrospray ionization mode with a spray voltage of 3.5 kV. Nitrogen was used as sheath, auxiliary, and sweep gas at flow rates of 49, 12, and 2 (arbitrary units), respectively. Chromatographic separation conditions were set via a gradient elution program: 0 to 1 minute, 0% to 10% B; 1 to 3.5 minutes, 10% to 35% B; 3.5 to 9 minutes, 35% to 50% B; 9.0 to 9.1 minutes, 50% to 0% B; and 9.1 to 11 minutes, 0% B. The total program runtime was 11 minutes. Data acquisition and processing were carried out using Thermo Scientific Xcalibur version 4.4. The detailed procedure for analysis by UHPLC-MS has been previously delineated.[18]

Statistical Analysis

Considering the smaller sample size and less likelihood of normal distribution of the data, nonparametric tests were performed to evaluate the differences between the groups (infection vs noninfection vs sham) at different time points. The Kruskal–Wallis test initially was used to evaluate the differences between the 3 groups (which showed overall difference between the study groups). When the Kruskal–Wallis test showed a significant difference between the groups at any given time point, a subsequent Wilcoxon rank-sum test was performed between the infection versus noninfection, infection versus sham, and noninfection versus sham groups to identify if the difference was between all 3 groups or between 1 or 2 groups only. All the analysis was done using SAS 9.4 software (SAS Inc) at 95% confidence level.

RESULTS

Using an inoculation dose of 40 mL solution containing 12×10^8 cfu of MSSA, we developed infection in 64% of the treated pigs. Those that developed radiographic evidence of infection and had evidence of MSSA present in their BAL were referred to as the MSSA infected group (n = 9), whereas those that were inoculated with MSSA but did not develop radiographic changes or positive cultures were referred to as the noninfected group (n = 5). Pigs that were inoculated with no bacteria were referred to as the Sham group (n = 8).

After inoculation, the pigs were monitored for clinical signs of pneumonia. Review of veterinary records from the postoperative monitoring period revealed pigs in the MSSA infected group started to develop clinical signs indicative of infection starting on PODs 1 and 2. Pigs within this group were noted to have poor appetite and lethargy, and require antipyretic medications by the veterinary care staff. Pigs within the MSSA noninfected group intermittently required antipyretic medication in the immediate postoperative period but were not reported to have sustained lethargy, loss of appetite, or persistent fevers after PODs 0 and 1. Pigs in the Sham inoculation group were not reported to show signs of lethargy or loss of appetite, or require antipyretic medication.

Pigs within the MSSA infected group developed consolidations on chest radiographs that persisted over the 9-day postoperative monitoring period. Representative radiographs from an infected pig on POD 9 is shown in Figure 1, *A*. All of the pigs in the infected group developed consolidations by POD 6, whereas 3 of the 9 pigs had developed consolidations by POD 3, and all still had consolidations at the termination of the experiment on POD 9. Pigs from both the MSSA noninfected group and Sham groups failed to develop consolidations on chest radiographs throughout the 9-day postoperative monitoring period, with representative radiographs shown in Figure 1, *B* and *C*.

Cultures grown from BAL samples of the MSSA-infected group demonstrated increasing counts of *S. aureus* over the 9-day postoperative period (Figure 2). Cultures grown from BAL samples of the MSSA noninfected group demonstrated some initial growth of *S. aureus,* but this eventually cleared over the 9-day postoperative period. Cultures grown from BAL samples of the Sham group failed to demonstrate growth of *S. aureus* on agar plates. There was a statistically significant increase in the amount of *S. aureus* recovered from the infected group at POD 3 relative to both the noninfected and sham groups ($P < .05$), and a significant difference at POD 6 and POD 9 ($P < .01$).

Breath Analysis of Inoculated Pigs

Mass spectroscopy analysis of the recovered breath from these pigs revealed values for 38 distinct carbonyl compounds. Analysis demonstrated 1 carbonyl compound (2-pentenal) increased 10-fold over the span of the experiment (Figure 3). The average values of 2-pentenal (C_5H_8O) were 0.0294 nmol/L on POD 0 before MSSA inoculation and then increased to an average of 0.3836 nmol/L on POD 9 ($P < .05$). The noninfected pigs showed a slight increase in the amount of 2-pentenal initially, which then returned to baseline levels as the experiment progressed. The sham inoculated pigs showed no significant change in this compound over the same time frame. There was a statistically significant elevation of the amount of 2-pentenal in the infected group relative to the sham group on POD 6 ($P < .02$) and a statistically significant elevation in the infected group relative to both the noninfected and sham group on POD 9 ($P < .01$).

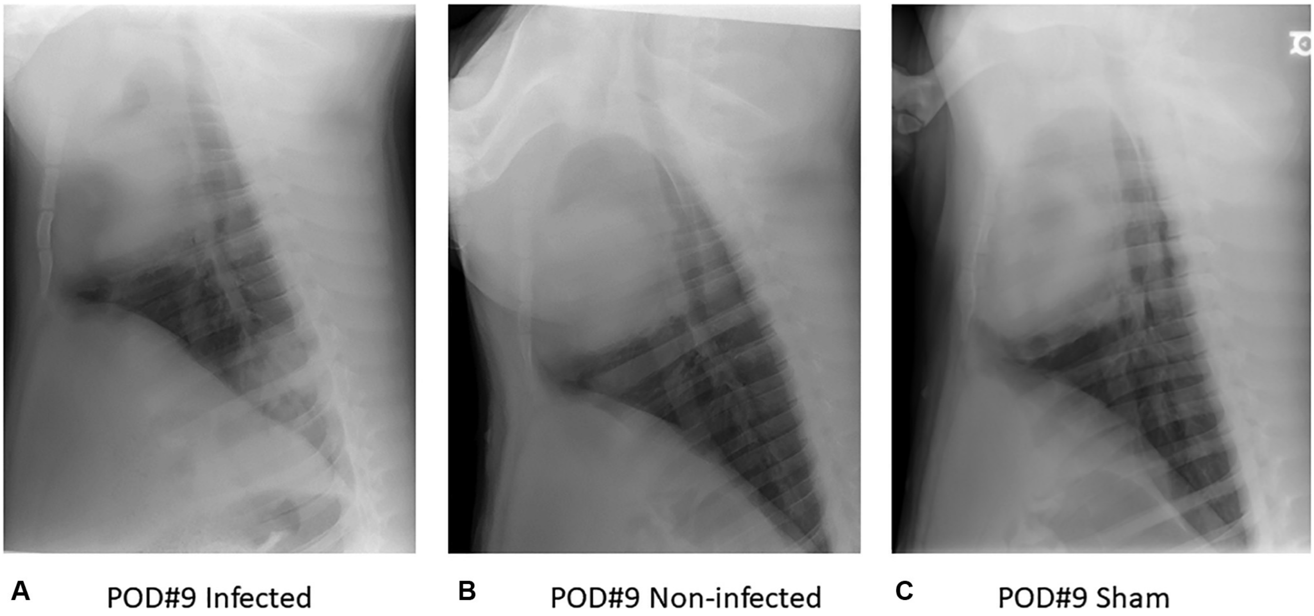

FIGURE 1. Chest x-rays of MSSA infected (A), MSSA inoculated but not infected (B), and Sham (C) inoculated pigs. *POD,* Postoperative day.

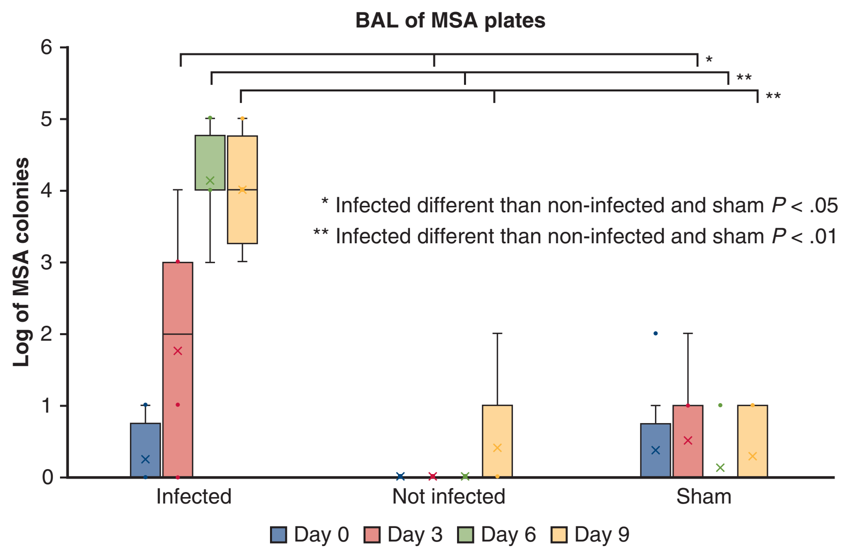

FIGURE 2. Growth of BAL from MSSA infected, uninfected, and Sham inoculated pigs. BAL taken from MSSA or Sham inoculated pigs was plated to quantify bacterial growth and graphed according to the logarithm of colony forming units grown. There was a statistically significant difference between the number of bacteria grown from the infected pigs and both the noninfected pigs and the sham inoculated pigs at POD 3 ($P < .05$), as well as a larger difference at POD 6 and POD 9 ($P < .01$). For the box-and-whisker plots, the *lower* and *upper borders* of the box represent the 25th and 75th percentiles, the *middle horizontal line* represents the median, and the *lower* and *upper* whiskers represent the minimum and maximum values of nonoutliers. *BAL,* Bronchoalveolar lavage; *MSSA*, methicillin sensitive *Staphylococcus aureus*.

None of the other captured compounds demonstrated a clear pattern of increase in the presence of infection. Butanone was one of these compounds, a biomarker that our group had previously demonstrated in the breath of human patients with lung cancer.[19] Figure 4 demonstrates the quantification of butanone over the course of the experiment in the infected, noninfected, and sham inoculated animals, demonstrating no significant differences between any group at any timepoint.

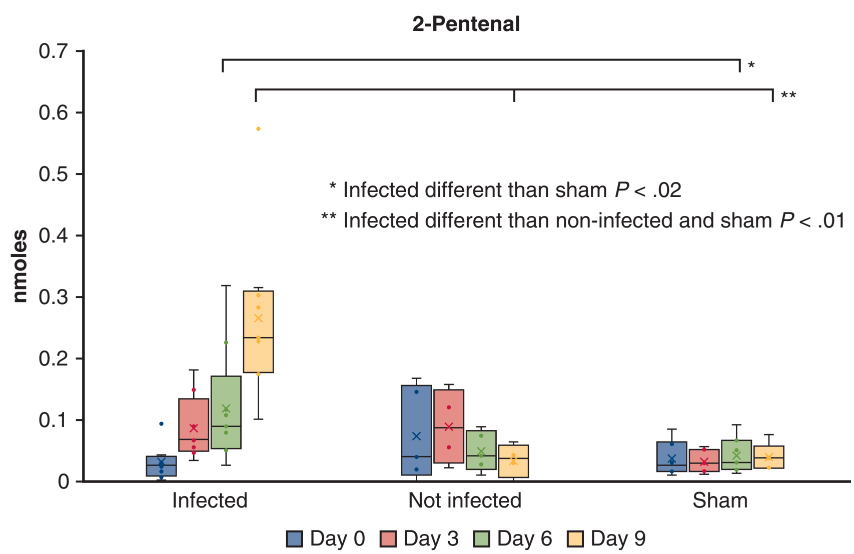

FIGURE 3. Levels of 2-pentenal in MSSA infected, uninfected, and Sham inoculated pigs. Comparison of the amount of 2-pentenal in nanomoles recovered from the breath of MSSA infected, MSSA inoculated but uninfected, and Sham inoculated pigs at several time points after inoculation. Amount of 2-pentenal recovered from the infected pigs at POD 6 was significantly ($P < .02$) higher than for the sham inoculated pigs. At POD 9, the amount of 2-pentenal was significantly ($P < .01$) higher than in the noninfected and sham inoculated pigs. For the box-and-whisker plots, the *lower* and *upper borders* of the box represent the 25th and 75th percentiles, the *middle horizontal line* represents the median, and the *lower* and *upper* whiskers represent the minimum and maximum values of nonoutliers.

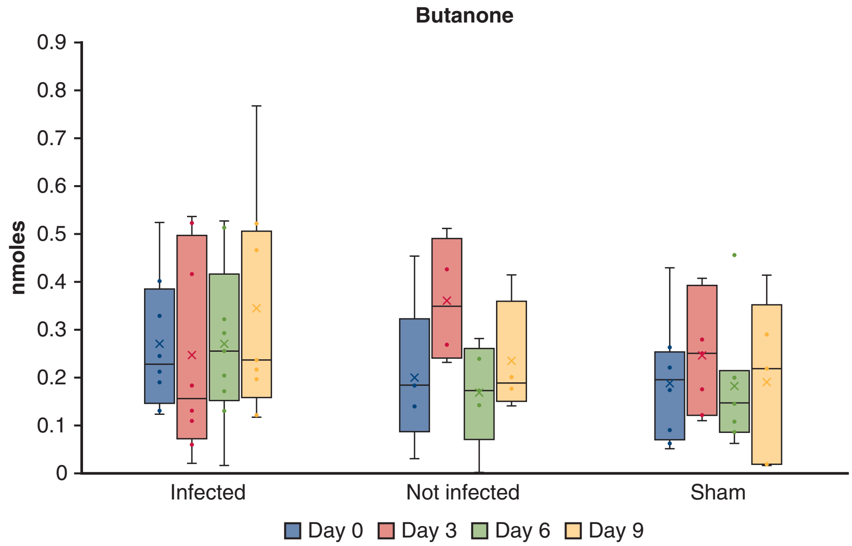

FIGURE 4. Levels of butanone in MSSA infected, uninfected, and Sham inoculated pigs. Comparison of amount of butanone in nanomoles recovered from the breath of MSSA and Sham inoculated pigs at several time points after inoculation. There were no significant differences between any group at any time-point. For the box-and-whisker plots, the *lower* and *upper borders* of the box represent the 25th and 75th percentiles, the *middle horizontal line* represents the median, and the *lower* and *upper* whiskers represent the minimum and maximum values of nonoutliers.

To determine the utility of 2-pentenal as a marker for the presence of pneumonia, we needed to establish a threshold for considering the amount of 2-pentenal to be abnormal. We determined the average amount of 2-pentenal present at any time point in the sham inoculated pigs (0.036995 nmoles) and the SD of this value (0.023612). We then defined a "positive" marker as any value that was greater than 2 SDs above the average value (>0.084219 nmoles). By using this cutoff as our criteria for a positive or negative marker, on POD 3, 5 of 9 of the infected pigs had a positive 2-pentenal marker, and 11 of 13 of the noninfected and sham pigs had a negative marker, yielding a sensitivity of 55% and specificity of 84% at POD 3. At POD 6, the sensitivity improved to 88%, and the specificity increased to 92%. Finally, at POD 9, the sensitivity and specificity were both 100%. All 9 infected pigs had a positive 2-pentenal marker, whereas none of the noninfected or sham pigs had a positive marker.

DISCUSSION

This study demonstrates that we were able to successfully develop a clinical pneumonia in adult Yorkshire pigs. Using this model, we were able to demonstrate an increase in levels of 2-pentenal in the breath samples of infected pigs. There have been several prior studies examining the "breath fingerprint" of volatile compounds in small animal models via mass spectroscopy that identified patterns of carbonyls that correlated with infection, although they did not focus on specific markers.[20-23] Although analysis of exhaled breath appears to be a promising noninvasive tool for the diagnosis of pneumonia in animal models, the clinical relevance in humans has yet to be established. A total of 3 human studies have looked at patterns of volatile organic compound profiles on intensive care patients with ventilator-associated pneumonia, all of which demonstrated a subset of compounds that correlated with pneumonia, but with relatively poor sensitivity and specificity.[24-26] Of note, 2-pentenal was not included in the compounds analyzed in those studies. Within these human studies, there are several potential confounding variables; it is unclear how variety in diet or environmental factors, for example, can alter the profile of exhaled carbonyl compounds. Our approach is distinct from both these prior animal and human studies in that it evaluates a specific marker in a quantifiable manner, in the controlled environment of a large animal model, where the diet and environmental exposures can be strictly controlled.

Although we only limited our infectious agent to a single species, *S. aureus*, during this study, there are a number of infectious agents that are capable of causing pneumonia in the clinical setting. As reported in the literature, changes within concentrations of volatile compounds within a host's exhaled breath may represent changes in metabolic processes occurring within the host as a result of the host's response to inflammation resulting from microbial infection or as a direct result of infectious agent.[14] Going forward, this model can be used to investigate pneumonia caused by other microbial pathogens. Because our study was limited to only a single infectious agent, we are unable to determine if 2-pentenal is a universal biomarker in the setting of pneumonia or if the increase in concentration of 2-pentenal correlates only with infection by *S. aureus*. We plan to expand this pig model to evaluate other common organisms associated with pneumonia, to see if this marker is associated with infection in general or is specific to this organism.

Study Limitations

A limitation of the current work is that we are only able to obtain breath samples and radiographs on pigs while they are sedated. Thus, we are unable to monitor for changes in radiographs and 2-pentenal concentrations on a daily basis due to safety of the animal and availability of resources. Ultimately, it would be beneficial to compare the development of infection more granularly with the increase in concentration of 2-pentenal.

CONCLUSIONS

Pneumonia remains a significant source of morbidity and mortality in the cardiothoracic patient population. Early diagnosis and initiation of adequate antibiotic therapy are essential in treating pulmonary infection and minimizing complications from the disease. There are many potential advantages provided by using a rapid and noninvasive method of analyzing exhaled breath for volatile organic compounds to make the diagnosis of pneumonia in the clinical setting, although further studies are needed to see if these findings can be extended beyond the use in this model. We are initiating a study in intubated patients at our facility to determine if our technology can identify markers that correlate with ventilator-associated pneumonias.

Conflict of Interest Statement

Drs van Berkel and Fu are founding members of Breath Diagnostics, Inc. All other authors reported no conflicts of interest.

The *Journal* policy requires editors and reviewers to disclose conflicts of interest and to decline handling or reviewing manuscripts for which they may have a conflict of interest. The editors and reviewers of this article have no conflicts of interest.

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