

# Development and implementation of enzymatic and volatile compound-based approaches for instantaneous detection of pathogenic *Staphylococcus aureus*

Divya Nori<sup>1</sup>, Evani Patel<sup>2</sup>, Amanda Martinez<sup>1</sup>

<sup>1</sup>Milton High School, Milton, Georgia

<sup>2</sup>Kennesaw Mountain High School, Kennesaw, Georgia

## SUMMARY

*Staphylococcus aureus* (*S. aureus*) has a mortality rate of up to 30% in developing countries. In contrast, the mortality rate of *S. aureus* in the U.S. is less than 5%. One reason for the six-fold increase in fatality in underdeveloped communities is lack of access to a rapid, accurate, and user-friendly diagnostic method to enable timely detection of these infections. The purpose of this experiment was to determine if enzymatic and volatile compound-based approaches would perform more quickly in comparison to existing *S. aureus* diagnostic methods and to evaluate these novel methods on accuracy. Further, we sought to implement them in a reusable 3D-printed instantaneous detection device. A combination of droplet transmission tests and direct contact tests were developed and evaluated to create a comprehensive method by which individuals can diagnose their condition as pathogenic *S. aureus*, incorrect use of device, or another infection. We hypothesized that the approaches developed in this study would perform more quickly than existing approaches. Ultimately, our device provided results in less than 30 seconds, which is much quicker than existing methods that take anywhere from 10 minutes to 48 hours based on approach. Statistical analysis of accuracy provides preliminary confirmation that our device based on enzymatic and volatile compound-based approaches can be an accurate and time-efficient tool to detect pathogenic *S. aureus*.

## INTRODUCTION

*Staphylococcus aureus*, hereby referred to as *S. aureus*, is the second-most common healthcare-associated infectious bacteria (1). Pathogenic *S. aureus* can infect many organs, the most common being the respiratory tract. *S. aureus* is a leading cause of death in hospitalized patients, with a mortality rate of up to 30% in underdeveloped communities (2). Pathogenic strains are often the cause of ventilator-associated pneumonia or a bacterial infection following influenza, both of which can be lethal conditions if not detected quickly (3). For *S. aureus*, respiratory droplets are

the primary mode of transmission, presenting a potential path by which a solution can be created. The ability to diagnose a respiratory tract staph infection in a cost-effective, self-administrable, and time-efficient manner is critical to reducing the mortality rate in developing countries. Current diagnostic methods include the swabbing and incubation of respiratory tract cultures, but results from these traditional tests take up to 48 hours to determine a definitive diagnosis (4). However, some rapid diagnostic testing methods have been researched in prior literature.

Polymerase chain reaction (PCR) is one method that has been pursued for potential early detection of *S. aureus* in the respiratory tract. The genetic biomarker that provides *S. aureus* with pathogenic properties is amplified and can therefore be more easily detected (5). However, PCR is not easily accessible in the developing world, and is not a viable diagnostic approach in patients' homes or smaller clinics due to the need for lab access (6). A rapid diagnostic method developed for *S. aureus*, known as the "rapid staph test" employs a latex fixation test and a lateral flow test. The latex fixation test or latex agglutination is used in the identification of microorganisms. If *S. aureus* antibodies are present, latex beads covered with antigens will firmly clump together to form a mass. The lateral flow test is more widely used, and if *S. aureus* antibodies are present when a sample is applied to nitrocellulose film, a visible line will form. However, while these tests are more accessible than PCR, they are not widely available in developing countries and are not reusable (7). Creating a diagnostic method that could be used more than once would allow for an entire community in a developing country to use a single test device. To bridge the gap seen in prior literature, four biochemical tests were assessed for time-efficiency and accuracy in this study. The tests were evaluated individually in flasks and later evaluated together within the device to ensure efficacy of the diagnostic tool.

The four biochemical tests evaluated are cost-effective, compound-based, and easily interpretable, making them unique from previous tests. Two of the tests, the calcium hydroxide test and hydrogen sulfide test, are droplet-based, meaning that the result will be displayed when the user exhales into the device. The remaining two tests, the hyaluronidase test and catalase test, are direct contact approaches meaning

that they are intended to analyze a sample that the user takes from the back of their throat using a sterile swab which is provided to the user with the device. Both testing methods ensure that patients in developing nations would not need additional equipment to perform the tests, and the patients could easily administer it themselves. The calcium hydroxide test was chosen to indicate to the user whether they are using the device correctly. To ensure that the gas sample is not contaminated by the outside air and is primarily the user's breath, the calcium hydroxide test detects the presence of carbon dioxide. This is because exhaled air consists of 4% to 5% carbon dioxide by volume which is a 100-fold increase over the amount of carbon dioxide in the air (8). Aqueous calcium hydroxide solution reacts with carbon dioxide gas to produce carbonic acid. Phenolphthalein indicator, a chemical compound that is colorless in acidic solutions and pink in basic solutions, turns clear when in contact with carbonic acid, providing a colorimetric indication of proper device use.

Hyaluronidases are a family of enzymes that breakdown the substrate hyaluronate. Several microorganisms have genes that encode hyaluronidase, including bacteria in the *Bacteroides*, *Treponema*, *Clostridium*, and *Propionibacterium* genera. Species in these genera can be found in the human gastrointestinal tract, intestines, or skin, but the only bacteria present in the human respiratory tract that produce hyaluronidases are *S. aureus*, *Streptococcus pyogenes*, and *Streptococcus pneumoniae* (9). Detecting the hyaluronidase enzyme would establish the presence of one of these three bacterial species, providing the rationale for the development of this test. When hyaluronidases come into contact with hyaluronic acid, hyaluronate is degraded into an oligosaccharide (10). If an oligosaccharide is produced in the enzyme reaction product, a chemical indicator for simple sugars such as Benedict's solution can detect the presence of hyaluronidase and establish that one of the three bacteria is present. Benedict's solution changes from blue to green when in the presence of a sugar polymer such as an oligosaccharide. The user is instructed to not eat anything for three hours before swabbing the back of their throat for this test so that traces of extraneous sugar are not present. This would help ensure that the activity of hyaluronidase is being detected and lower the chance of a false positive. While this method has not been used as an enzymatic assay for hyaluronidase in the past, we attempted to provide evidence for the efficacy of this approach due to its easy interpretability for users who are self-administering the test.

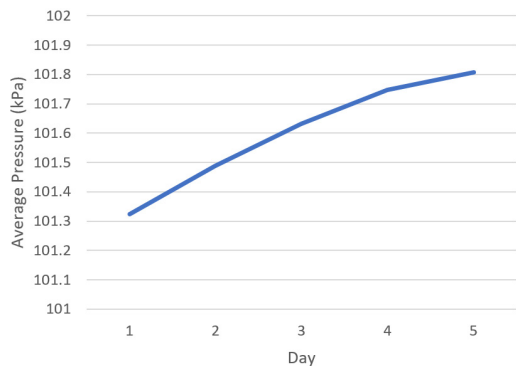
The catalase test was chosen as one of the contact-based tests for this device to differentiate between *S. aureus*, *S. pyogenes*, and *S. pneumoniae* (after a positive result for the hyaluronidase test). *S. aureus* is catalase positive, while *S. pyogenes* and *S. pneumoniae* are catalase negative because all streptococci lack the enzyme catalase (11-12). Currently, *S. aureus* is speculated to use hyaluronidase as a virulence factor. However, since this has not been supported with sufficient evidence, a more fool-proof approach to identify

the pathogenicity of the bacterial strain is necessary (13). Pathogenicity must be established to attribute the positive result of the hyaluronidase test to a *S. aureus* infection and not a hyaluronidase enzyme encoded by human cells or non-pathogenic *S. aureus* (14-15). Pathogenic staphylococcus strains release hydrogen sulfide-based cytotoxins called volatile sulfur compounds (VSCs). While for some species of bacteria both the non-pathogenic and pathogenic strains produce hydrogen sulfide, it has been established that both dimethyl-disulfide and methanethiol are released by pathogenic *S. aureus* and not by non-pathogenic strains (16-17). The hydrogen sulfide test turns a copper sheet from copper colored to black in the presence of dimethyl-disulfide or methanethiol. The color change is based on the reaction between the VSC and copper that produces copper sulfide and copper oxide (18-19). When the results from the calcium hydroxide test, hyaluronidase test, catalase test, and hydrogen sulfide test are interpreted in conjunction, a patient's condition can be identified as pathogenic *S. aureus*, incorrect use of device, or a different infection (result cannot be determined with this device). The two direct contact tests (catalase and hyaluronidase) and two breath-based tests (calcium hydroxide and hydrogen sulfide) were developed to create a diagnostic process that will perform accurately and efficiently when hosted in a 3D-printed reusable device.

The central aims of this study were to determine the plausibility of using enzyme biomarkers and volatile compounds released by bacteria to enable early detection, engineer a device applying these findings to detect *S. aureus* in a patient's respiratory tract, and compare the results to existing diagnostic approaches. We hypothesized that the approaches developed in this study would perform more quickly than existing approaches. The null hypothesis can be rejected in favor of this alternate hypothesis because after repeated testing, the device was able to perform in under 30 seconds which is less than the time taken to perform the rapid staph test (10 – 20 minutes) and the *S. aureus* culture test (24 – 48 hours). After Fisher's exact test analysis, a significant difference was observed in hyaluronidase test results between *S. aureus* and *Staphylococcus epidermidis*, hereby referred to as *S. epidermidis*. Non-pathogenic *S. aureus* released a gas (verified by air pressure readings) whose chemical composition triggered a negative result for the hydrogen sulfide test. Therefore, this study provides preliminary confirmation that gaseous metabolites and enzymes can be used to identify certain bacterial infections. Our hypothesis was supported because volatile compound and enzymatic approaches performed in less time than existing methods.

## RESULTS

Before assessing the effectiveness of the diagnostic tests, throughout the bacterial incubation process, an air pressure sensor to monitor the production of gas by *S. aureus* and verify that gaseous metabolites were being released. The measured air pressure inside the flask increased over time from 101.325



**Figure 1.** Average air pressure in the Erlenmeyer flasks over time. Each flask contained 50 mL of *S. aureus* broth, and the rate of gas production remained fairly constant as time went on.

kPa to 101.807 kPa (Figure 1). The bacteria produced gas in a way that closely resembled a linear pattern. The linear gas trend was maintained throughout the gas collection process, indicating that the gas production rate remained constant.

The calcium hydroxide test and hyaluronidase test were then evaluated individually in flasks, and the rationale for this portion of the experiment was to determine the optimal conditions for each test. The metric for the calcium hydroxide test was volume of test solution.

We evaluated a range of solution volumes to determine which exhibits the quickest color change (Table 1). A direct correlation between solution volume (volume of calcium hydroxide solution onto which the user breathes) and average time in seconds taken to reach a color change was observed. The lowest time taken to reach a color change was 5.1 seconds with a solution volume of 2 mL. Other factors such as room conditions were controlled for, and the samples of healthy breath were taken from the two student researchers, eliminating the need for outside human participants. The researchers blew onto the liquid as they would normally exhale. After identifying the optimal test solution volume of 2 mL, our next experiment was to confirm the observed color change of the calcium hydroxide test when breath is released correctly (Figure 2). The beakers initially contained 2 mL of bright pink solution consisting of H<sub>2</sub>O, Ca(OH)<sub>2</sub>, and phenolphthalein. After reacting with carbon dioxide, the solution became transparent (Figure 2).

Next, we evaluated metrics for the hyaluronidase test

**Table 1.** Time taken to reach a color change from pink to clear for the calcium hydroxide test across different solution volumes.

Test	Sample	Volume of Test Solution (mL)	Average time in seconds (across four trials)
Calcium Hydroxide	Healthy body (breath)	25	64.2
Calcium Hydroxide	Healthy body (breath)	10	28.6
Calcium Hydroxide	Healthy body (breath)	5	8.3
Calcium Hydroxide	Healthy body (breath)	2	5.1

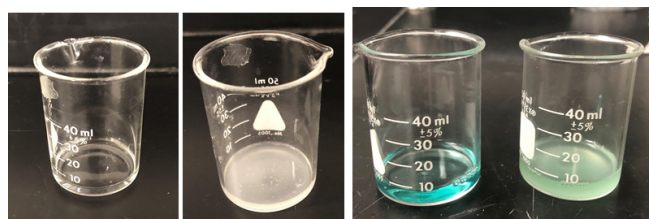


**Figure 2.** Color change observed due to the reaction of calcium hydroxide and carbon dioxide. Left is before reaction with the addition of phenolphthalein indicator, whereas right is after reaction.

for *S. aureus*, a strain encoding the hyaluronidase enzyme, and *S. epidermidis*, a strain not encoding the hyaluronidase enzyme (Table 2). We collected data for several test solution compositions (solvent-solute ratios), and the hyaluronidase test performed the quickest when the solvent-solute ratio was 3:1 with a time of 6.5 seconds (Table 2). The solvent was hyaluronic acid, and the solute was Benedict's solution, which was used to detect the presence of an oligosaccharide when hyaluronic acid was degraded by hyaluronidase. This shows that in the device, the volume of hyaluronic acid should be three times the volume of Benedict's solution for the most time-efficient results. This optimal 3:1 acid-indicator ratio was used to confirm the hyaluronidase test's color change with *S. aureus* and *S. epidermidis*. *S. epidermidis* failed to react, so the color remained bright blue/teal (Figure 3). *S. aureus* did

**Table 2.** Time taken to reach a color change from blue to green for the hyaluronidase test across different ratios of hyaluronic acid and Benedict's solution in the test solution.

Test	Sample	Solvent-Solute Ratio Solution (mL)	Average time in seconds (across four trials)
Hyaluronidase	<i>S. aureus</i> (culture)	5 to 1	9.4
Hyaluronidase	<i>S. aureus</i> (culture)	3 to 1	6.5
Hyaluronidase	<i>S. aureus</i> (culture)	1 to 1	7.9
Hyaluronidase	<i>S. epidermidis</i> (culture)	5 to 1	No Reaction
Hyaluronidase	<i>S. epidermidis</i> (culture)	3 to 1	No Reaction
Hyaluronidase	<i>S. epidermidis</i> (culture)	1 to 1	No Reaction



**Figure 3.** The first panel (starting from the left) shows the result of the catalase test after failing to react with *E. faecalis*, and the second panel shows the result of the catalase test after reaction with *S. aureus*. The third panel shows the result of the hyaluronidase test after failing to react with *S. epidermidis*, and the fourth panel shows the result of the hyaluronidase test after reaction with *S. aureus*.

react, and the color of the solution changed from bright blue to pale green (Figure 3).

We next evaluated the catalase and hydrogen sulfide tests individually (Table 3). For the catalase test, the average time taken for a color change with the *S. aureus* culture was 4.5 seconds (Table 3). The original hydrogen peroxide solution was transparent, and when the *S. aureus* culture reacted, a milky white color resulted (Figure 3). However, the *E. faecalis* did not react with the hydrogen peroxide solution, so the beaker remained transparent. The hydrogen sulfide test did not result in a reaction because tested bacterial strains were non-pathogenic (Table 3).

We then used CAD software to develop device design prototypes. Our final design involves an opening through which the patient can easily breathe into the compartments, and this opening is designed to streamline the air into the desired locations (Figure 4). The bottom portion of the device contains four compartments, one for each test, and the top and bottom portions of the device are sealed together in an airtight fashion. Using this device, we performed a series of 30 final trials (Figure 5). These 30 trials consist of 10 *S. aureus* trials, 10 *E. faecalis* trials, and 10 *S. epidermidis* trials, and all of the tests were conducted using non-pathogenic bacteria because pathogenic bacteria were not available to us.

Using data from these final trials, we calculated the Fisher's exact statistic value. We began with the hyaluronidase test, and the null hypothesis was that there is no significant difference in hyaluronidase test results between *S. aureus* and *S. epidermidis* within the device. The alternate hypothesis was that there is a significant difference in hyaluronidase test results between *S. aureus* and *S. epidermidis*. If the computed Fisher's exact test value was less than a *p*-value of 0.01, the null can be rejected in favor of the alternate hypothesis. The device color sensor detected a pale green color for nine of the non-pathogenic *S. aureus* trials when hosted in the device and detected a blue color for one of the non-pathogenic *S. aureus* trials (Table 4). A blue test solution was detected for all 10 of the *S. epidermidis* trials by the device color sensor. The Fisher's exact test statistic value was 0.0001 which shows that the result was significant at *p* < 0.01. The null hypothesis was rejected, displaying that the hyaluronidase test was likely effective within the device.

**Table 4. Contingency table for hyaluronidase test, used in the computation of the Fisher's exact test statistic.**

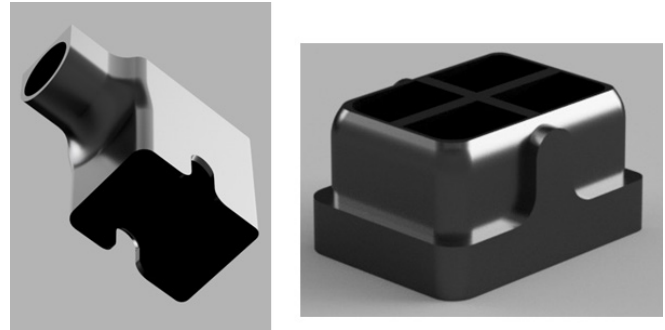
	Non-Pathogenic <i>S. aureus</i>	Non-Pathogenic <i>S. epidermidis</i>
Hyaluronidase Positive	9 samples	0 samples
Hyaluronidase Negative	1 sample	10 samples

**Table 5. Contingency table for catalase test, used in the computation of the Fisher's exact test statistic.**

	Non-Pathogenic <i>S. aureus</i>	Non-Pathogenic <i>E. faecalis</i>
Catalase Positive	8 samples	0 samples
Catalase Negative	2 samples	10 samples

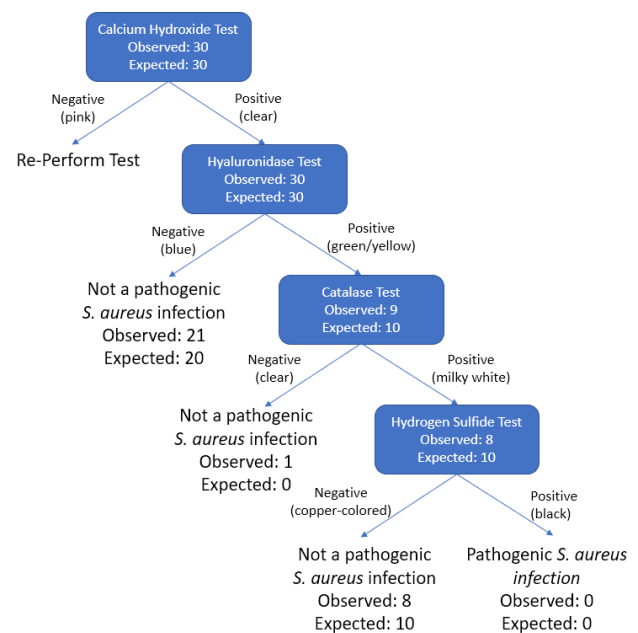
**Table 3. Metrics from the catalase and hydrogen sulfide tests.**

Test	Sample	Average time in seconds (across four trials)
Catalase	<i>E. faecalis</i> (culture)	No Reaction
Catalase	<i>S. aureus</i> (culture)	4.5
Hydrogen Sulfide	<i>S. aureus</i> (gas)	No Reaction



**Figure 4. CAD design created to 3D-print the physical device.** The main features include a straw-like design to streamline the breath, an airtight lid, and four compartments for test liquids.

For the catalase test, the null hypothesis was that there is no significant difference in a catalase test results between *S. aureus* and *E. faecalis* within the device. The alternate hypothesis was that there is a significant difference in catalase test results between *S. aureus* and *E. faecalis*. The color sensor detected a white color for eight of the *S. aureus* trials and detected no color (transparent) for two of the trials (Table 5). The color sensor detected no color (transparent)



**Figure 5. Flow chart of possible end results based on combinations of test results.** The observed and expected counts for the final 30 trials conducted within the device are also shown.

for all 10 of the *E. faecalis* trials. The Fisher's exact statistic value was 0.0007, showing that the result is significant at  $p < 0.01$ . The null hypothesis can be rejected in support of the alternate hypothesis, showing the catalase test's efficacy within the device.

## DISCUSSION

The aim of this work was to determine the plausibility of using enzyme biomarkers and volatile compounds released by bacteria to enable early detection and engineer a device to detect *S. aureus* in a patient's respiratory tract. These approaches were then compared to existing approaches based on time-efficiency. This study provides preliminary confirmation that gaseous metabolites and enzymes can be used to identify certain bacterial infections.

The change in pressure demonstrates the accumulation of gas that is released by *S. aureus*, containing the molecules necessary to detect the infection. The stark color change in the calcium hydroxide test indicates that the test could easily identify if a patient were breathing into the device correctly and if the necessary amount of breath were blown into the device. The average time for a distinct color change was 5.1 seconds for a 2 mL trial, making this the optimal volume of liquid to be administered in the compartment for a rapid diagnosis.

The hyaluronidase enzymes present in the *S. aureus* reacted with hyaluronic acid to form the oligosaccharide that turned Benedict's solution from blue to green. Future studies could explore how color change could be maximized - for example, if there was a heating coil in the device to heat Benedict's solution - which may provide a more noticeable color difference. Hyaluronic acid solutions of higher concentration could also provide more easily detectable changes during tests. Evaluation of the hyaluronidase test was conducted using *S. aureus* and *S. epidermidis* cultures, and it is important to note that in these trials, the number of bacterial cells transferred to the test solution likely outnumbered those in a human patient. While evaluation in this study was conducted using pure cultures, in the future, the device could be tested with healthy and infected individuals to observe its efficacy within a real human body. Before human trials can be conducted, the threshold to a positive hyaluronidase result (i.e. the number of colonies needed to trigger a color change from blue to green) could be established.

When the catalase test was performed on *S. aureus* bacteria, the result was a milk white solution. This change from a clear solution to a milky solution occurs because *S. aureus* contains catalase, which detoxifies hydrogen peroxide. *E. faecalis* displayed a negative result, and because it is genetically similar to *S. pyogenes*/*S. pneumoniae* in that they do not synthesize heme and therefore lack the catalase enzyme, this test serves to classify whether *S. aureus* has been identified if the user's hyaluronidase test is positive (20). If the hyaluronidase test is negative, a conclusion cannot be drawn from the catalase test, but if both the hyaluronidase

and catalase tests are positive, *S. aureus* has been identified. However, pathogenicity cannot be established without the hydrogen sulfide test.

Analysis using Fisher's exact test displays the efficacy of the hyaluronidase and catalase tests within the actual device. While testing with non-pathogenic *S. aureus*, *E. faecalis*, and *S. epidermidis* shows that the hydrogen sulfide test displayed a negative result when in contact with non-pathogenic strains of bacteria, further testing is required to ensure that the proper color change occurs when in contact with pathogenic *S. aureus*. The pathogenic strains were not available in our lab for testing. Future studies could examine *S. pyogenes* or *S. pneumoniae* in comparison to *S. aureus* to certify that *S. aureus* can be differentiated from these bacteria.

After repeated testing with all four direct contact and droplet transmission approaches hosted within the device, a result was reached in under 30 seconds which is less than the time taken to perform the rapid staph test (10–20 minutes) and the *S. aureus* culture test (24–48 hours). This supports our initial hypothesis that a test based on enzymatic and volatile compound-based approaches will perform more quickly than existing techniques and provides evidence supporting the plausibility of these approaches for the accurate diagnosis of *S. aureus* infections in the respiratory tract.

## METHODS

Mannitol salt agar was prepared and poured into petri dishes as the base for bacterial growth. Samples of non-pathogenic *S. aureus*, *S. epidermidis* (negative control for hyaluronidase test), and *E. faecalis* (negative control for catalase test) were each swabbed onto dishes using an inoculating loop. These plates were incubated for five days at 37°C and were used in testing of the direct-contact tests (hyaluronidase and catalase). Incubation of *S. pyogenes* or *S. pneumoniae* was not permitted in our laboratory environment and hence alternative comparison species were used. In addition, to ensure the production of gaseous metabolites by *S. aureus*, three Erlenmeyer flasks each holding 50 mL of *S. aureus* broth were closed with rubber stoppers to collect gas released by the bacteria. These liquid cultures were grown at 35°C. In 24-hour increments, the pressure inside the flask was measured using an air pressure sensor. This process was repeated over the course of five days to track the production of gas by *S. aureus*. Five days were chosen as supposed to a shorter time frame to observe any change in the rate of gas production over a longer period.

To begin the calcium hydroxide test, limewater was prepared by adding 3 grams of  $\text{Ca(OH)}_2$  to 150 mL of distilled water. The limewater then rested for 24 hours to dissolve into a homogenous solution. Then, twelve drops of phenolphthalein indicator solution were added to 200 mL of distilled water along with 3 mL of lime water which turned the stock solution a magenta shade. This original solution had a pH of ~13.6 (extremely basic), and phenolphthalein changes

color between 8.2 and 10.0. Among the common acid-base indicators, phenolphthalein has the most basic pH range for a color change which is why it was chosen. The carbon dioxide must cause a drop of approximately 4 pH points before the solution changes from pink to clear. The stock solution was poured into 16 beakers: 4 with 25 mL, 4 with 10 mL, 4 with 5 mL, and 4 with 2 mL of stock solution.

The test was then administered in 16 trials between 2 subjects (the two researchers). A trial comprised of each subject blowing on to the solution, and each subject completed two trials for each volume. The subject would blow onto the solution until the solution changed from a magenta color to clear. These trials were then analyzed to find the average time in seconds to reach a color change depending on the volume of test solution to find the optimal volume that would be used in the detection device. Because accuracy did not decrease with smaller volumes, the optimal volume was chosen as 2 mL because it was the most time efficient. The color change from the calcium hydroxide is easy to detect and interpret for a user in a developing country with little medical knowledge, so the calcium hydroxide test was identified as the best positive control.

The next test developed was for the hyaluronidase enzyme. The components of the hyaluronidase test solution are hyaluronic acid and Benedict's solution. We measured 5 mL of 5.0 M hyaluronic acid into 24 different beakers, and for each set of 8 beakers, a different acid-indicator ratio was tested. The tested hyaluronic acid to Benedict's solution ratios were 5:1, 3:1, and 1:1. Benedict's solution, which is composed of sodium citrate, sodium carbonate, and copper (II) sulfate pentahydrate, changes color when in contact with simple sugars. This indicator was chosen as opposed to the Fehling's test or Tollens' test due to availability in our lab, although these solutions would all display a colorimetric indication of an oligosaccharide. Each set of 8 beakers was then divided into two groups, one group to test *S. aureus* bacteria and one group to test *S. epidermidis* bacteria. *S. epidermidis* was used as a negative control for the hyaluronidase test because its genome does not encode the hyaluronidase enzyme (21). The beakers in each group of four were labeled and corresponded to four petri dishes of *S. aureus* and four petri dishes of *S. epidermidis*. A sterile swab was used to take samples of the bacterial culture and insert it into the solution. Five swabs were taken from each petri dish and inserted into one beaker of each ratio. Using this method, every combination of ratio and bacteria type was tested four times, and time taken to reach a color change was measured for each ratio. While this is not an established method for determining the presence of hyaluronidase, the Fisher's exact test results show promise for the described approach.

The catalase test involved comparison of results from swabs of *E. faecalis* to swabs of *S. aureus*. To begin, a 3% concentration hydrogen peroxide solution was poured into eight beakers. The beakers were separated into two groups of four, one to test *S. aureus* and one to test *E. faecalis*.

The beakers corresponded to four petri dishes of *S. aureus* and four petri dishes of *E. faecalis*. *E. faecalis* was used as a negative control for the catalase test because it is a genetically close relative to *S. pyogenes* and *S. pneumoniae* (*S. pyogenes* and *S. pneumoniae* were not available at our lab to us) and cannot synthesize catalase without the presence of heme (22). A sterile swab was used to take a sample from each Petri dish, and each sample was inserted into a different beaker. Similar to the hyaluronidase test, the time taken to reach an observable change from transparent to translucent (milky white) was recorded and analyzed. The catalase test works because catalyze enzymes catalyze the decomposition of hydrogen peroxide into oxygen and water. The oxygen creates bubbles in the solution, making it a translucent color. If both the hyaluronidase and catalase tests are positive, *S. aureus* has been identified.

The final test, the hydrogen sulfide test, required four copper sheets, each cut to 1 cm by 0.75 cm. The sheets were then scrubbed with sandpaper and dusted off to remove any impurities that may lay on the copper's surface. The sheets were wiped and sprayed with acetone to be ready for the test. Much like the calcium hydroxide test, the effectiveness of breath-based administration was evaluated. Because hydrogen sulfide-based gases are denser than air, the gas released by non-pathogenic *S. aureus* was collected by inserting a gas syringe into an Erlenmeyer flask, ensuring that it was airtight. This gas retrieval process was repeated four times, and the gas produced by *S. aureus* bacteria was released onto the prepared copper sheet. Time taken for a potential color change was recorded. The gas syringe was used to mimic the force of a human breathing into the device. While a color change was not observed because non-pathogenic bacterial strains were unavailable, the presence of hydrogen sulfides are likely the only cause of a potential change in color of copper sheets in this use case because when in contact with regular air, copper takes years to exhibit a significant color change (23). Future experiments must be conducted to ensure that a color change to black occurs in the presence of gas released by pathogenic *S. aureus*.

The following steps were taken to create the physical device. A CAD design was created in TinkerCAD, and the design allows room for four solutions needed for the chemical tests, a water-proof compartment for the color sensor and Bluetooth module, a cylindrical apparatus through which the patient can easily breathe into the device, an airtight lid which can be easily removed to interpret LED results, clean compartments for solutions, and refill the device for reuse. A TCS230 Arduino-based color recognition system, HC-05 Bluetooth module, an Arduino Nano board, and LED lights were used to configure the electronic system embedded into the device. A color sensor emits a white light and monitors the red, green, and blue components of the light reflected by a substance using three receivers. The intensity of red, green, and blue light reflected enables the sensor to determine the color of the substance. In this way, potential color changes

from each test are detected. When a user receives the device, these electronic components will be integrated into the 3D-printed casing, costing less than 30 dollars for a device that can sustain an entire community. Based on the color of each of the test solutions, appropriate LED lights are activated, telling the patient their results for each test. The integration of a sensor and LED-based result interpretation removes potential human reading errors.

After the device creation process was complete, the final stock solutions as refined in the individual chemical test trials were poured into the appropriate compartments. We conducted 30 final trials to assess the overall functionality of the device. These trials consisted of 10 *S. aureus* trials, 10 *S. epidermidis* trials, and 10 *E. faecalis* trials. The direct contact tests (hyaluronidase and catalase) were conducted by taking a small sample of the respective culture from a petri dish using a sterile inoculating loop and placing it in the test solution hosted within a compartment in the device. The breath-based tests (calcium hydroxide and hydrogen sulfide) were conducted by collecting gas in Erlenmeyer flasks, following the same methods described initially to measure the pressure of gas released by *S. aureus*, and a sterile syringe was used to collect gas from the flask after 24 hours of incubation. The syringe was used to release the gas through the straw-like blow hole of the device to mimic a person breathing through the tube as designed.

#### ACKNOWLEDGEMENTS

We would like to thank Ms. Vaillancourt for her helpful feedback on the manuscript. Thank you to Mr. Lowry for his guidance and the Georgia Governor's Honors Program for materials.

**Received:** January 9, 2020

**Accepted:** October 24, 2020

**Published:** February 20, 2021

#### REFERENCES

1. Revelas Angela. "Healthcare-associated infections: A public health problem." *Nigerian Medical Journal*, vol. 53, no. 2, 2012, pp. 59-64. doi: 10.4103/0300-1652.103543.
2. Tong, Steven, *et al.* "Staphylococcus aureus infections: Epidemiology, pathophysiology, clinical manifestations, and management." *Clinical Microbiology Reviews*, vol. 28, no. 3, 2015, pp. 603-661. doi: 10.1128/CMR.00134-14.
3. Burnham, Jason, and Marin Kollef. "Prevention of Staphylococcus aureus ventilator-associated pneumonia: conventional antibiotics won't cut it." *Clinical Infectious Diseases*, vol. 64, no. 8, 2017, pp. 1089-1091. doi: 10.1093/cid/cix060.
4. Paul, Suzanne, *et al.* "Direct Detection of Staphylococcus aureus from Adult and Neonate Nasal Swab Specimens Using Real-Time Polymerase Chain Reaction." *The Journal of Molecular Diagnostics*, vol. 6, no. 3, 2004, pp. 191-196. doi: 10.1016/S1525-1578(10)60509-0.
5. Conterno, L.O., *et al.* "Real-time polymerase chain reaction detection of methicillin-resistant Staphylococcus aureus: impact on nosocomial transmission and costs." *Infection Control and Hospital Epidemiology*, vol. 28, no. 10, 2007, pp. 1134-1141. doi: 10.1086/520099.
6. Tauyoun, Ahmad, *et al.* "Democratizing Molecular Diagnostics for the Developing World." *American Journal of Clinical Pathology*, vol. 141, no. 1, 2014, pp. 17-24. doi: 10.1309/AJCPA1L4KPXBJNPG.
7. Koczula, Katarzyna, and Andrea Gallotta. "Lateral flow assays." *Essays in Biochemistry*, vol. 60, no. 1, 2016, pp. 111-120. doi: 10.1042/EBC20150012.
8. Ejaimi, Gamal, and Sittelnissa Saeed. "An Introduction to Airway Assessment and Management." *Annals of International Medical and Dental Research*, vol. 3, no. 1, 2017, pp. 1-7. doi: 10.21276/aimdr.2017.3.1.AN1.
9. Hynes, Wayne, and Sheryl Walton. "Hyaluronidases of Gram-positive bacteria." *FEMS Microbiology Letters*, vol. 183, no. 2, 2000, pp. 201-207. doi: 10.1111/j.1574-6968.2000.tb08958.x.
10. Payan, Elizabeth, *et al.* "Hyaluronidase degradation of hyaluronic acid from different sources: Influence of the hydrolysis conditions on the production and the relative proportions of tetra- and hexasaccharide produced." *International Journal of Biochemistry*, vol. 25, no. 3, 1993, pp. 325-329. doi: 10.1016/0020-711X(93)90620-T.
11. Torok, Estee, and Nick Day. "Staphylococcal and streptococcal infections." *Medicine*, vol. 33, no. 5, 2005, pp. 97-100. doi: 10.1383/medc.33.5.97.64964.
12. King, Katherine, *et al.* "Aerotolerance and Peroxide Resistance in Peroxidase and PerR Mutants of Streptococcus pyogenes." *Journal of Bacteriology*, vol. 182, no. 19, 2000, pp. 5290-5299. doi: 10.1128/jb.182.19.5290-5299.2000.
13. Ibberson, Carolyn, *et al.* "Staphylococcus aureus hyaluronidase is a CodY-regulated virulence factor." *Infection and Immunity*, vol. 82, no. 10, 2014, pp. 4253-4264. doi: 10.1128/IAI.01710-14.
14. Chao, Kinlin, *et al.* "Structure of human hyaluronidase-1, a hyaluronan hydrolyzing enzyme involved in tumor growth and angiogenesis." *Biochemistry*, vol. 46, no. 21, 2007, pp. 6911-6920. doi: 10.1021/bi700382g.
15. Man, Wing, *et al.* "The microbiota of the respiratory tract: gatekeeper to respiratory health." *Nature Reviews Microbiology*, vol. 15, no. 5, 2017, pp. 259-270. doi: 10.1038/nrmicro.2017.14
16. Filipak, Wojciech, *et al.* "Molecular analysis of volatile metabolites released specifically by Staphylococcus aureus and Pseudomonas aeruginosa." *BMC Microbiology*, vol. 12, no. 113, 2012. doi: 10.1186/1471-2180-12-113.
17. Peng, Hui, *et al.* "Hydrogen Sulfide and Reactive Sulfur Species Impact Proteome S-Sulfhydration and Global

- Virulence Regulation in *Staphylococcus aureus*." *ACS Infectious Diseases*, vol. 3, no. 10, 2017, pp. 744-755. doi: 10.1021/acsinfecdis.7b00090
18. Meldau, Dorothea, *et al.* "Dimethyl disulfide produced by the naturally associated bacterium *Bacillus* sp B55 promotes *Nicotiana attenuata* growth by enhancing sulfur nutrition." *The Plant Cell*, vol. 25, no. 7, 2013, pp. 2731-2747. doi: 10.1105/tpc.113.114744
  19. Tran, Thi, *et al.* "The atmospheric corrosion of copper by hydrogen sulphide in underground conditions." *Corrosion Science*, vol. 45, no. 12, 2003, pp. 2787-2802. doi: 10.1016/S0010-938X(03)00112-4.
  20. Yother, Janet, *et al.* "Genetics of Streptococci, Lactococci, and Enterococci: Review of the Sixth International Conference." *Journal of Bacteriology*, vol. 184, no. 22, 2002, pp. 6085-6092. doi: 10.1128/JB.184.22.6085-6092.2002.
  21. Post, Virginia, *et al.* "Comparative Genomics Study of *Staphylococcus epidermidis* Isolates from Orthopedic-Device-Related Infections Correlated with Patient Outcome." *Journal of Clinical Microbiology*, vol. 55, no. 10, 2017, pp. 3089-3103. doi: 10.1128/JCM.00881-17.
  22. Baureder, Michael, and Lars Hederstedt. "Genes Important for Catalase Activity in *Enterococcus Faecalis*." *PLOS One*, vol. 7, no. 5, 2012, pp. 6351-6356. doi: 10.1128/JB.184.22.6351-6356.2002.
  23. Leygraf, Christofer, *et al.* "The origin and evolution of copper patina colour." *Corrosion Science*, vol. 157, no. 2, 2019, pp. 337-346. doi: 10.1016/j.corsci.2019.05.025.

**Copyright:** © 2020 Nori, Patel, and Martinez. All JEI articles are distributed under the attribution non-commercial, no derivative license (<http://creativecommons.org/licenses/by-nc-nd/3.0/>). This means that anyone is free to share, copy and distribute an unaltered article for non-commercial purposes provided the original author and source is credited.