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David Watson Date

Quantification of *Streptococcus pneumoniae* in the Human Nasopharynx: Assay Validation Studies for the FDA

By

David Watson

Degree to be awarded: MPH

Hubert Department of Global Health

_____

Jorge E. Vidal, PhD
Committee Chair

Quantification of *Streptococcus pneumoniae* in the Human Nasopharynx: Assay Validation Studies for the FDA

By

David Watson

Bachelors of Arts in Sociology and Anthropology
St. Olaf College
2014

Thesis Committee Chair: Jorge E. Vidal, PhD

An abstract of
A thesis submitted to the Faculty of the Rollins School of Public Health of Emory University
in partial fulfillment of the requirements for the degree of
Master of Public Health in Global Health
2016

Abstract

Quantification of *Streptococcus pneumoniae* in the Human Nasopharynx:
Assay Validation Studies for the FDA
By David Watson

Introduction:

Diagnosing a case of pneumococcal pneumonia can be very difficult due to the drawbacks of the current gold-standard methods based on bacterial culture. Recently several new qPCR procedures have been developed to identify genes specific to the pneumococcus, making accurate identification easier. We set out to validate the new *lytA* assay for use in future clinical studies.

Methods:

The *lytA* assay was conducted as described in this report, with careful attention to quality control measures and strict adherence to predetermined acceptance criteria. In order to establish the performance characteristics of the *lytA* assay, a series of experiments were conducted. These experiments included a linearity study, a limit-of-detection study, a replication experiment, an interference study and a comparison of methods study. These experiments were designed to determine the assay's reportable range, analytical sensitivity, precision, analytical specificity, and accuracy, respectively.

Results:

In the linearity study linear regression provided an R^2 of 0.9941, indicating a strong linear relationship between assigned values of standard concentrations and values measured using the assay. In the limit-of-detection study the overall LOD for the assay was 4.28 copies/reaction. In the replication experiment the assay performed very reliably with low standard deviations in run and across runs. In the interference study, all replicates of the 29 organisms tested were not detected by the *lytA* qPCR assay, while TIGR4 positive controls were detected. Finally, in the comparison of methods study the *lytA* assay found all of the 40 *S. pneumoniae*-spiked specimens to be positive, matching the gold standard methods.

Discussion and Conclusion:

The experiments conducted in this study display the efficacy of the *lytA* assay and allow us to establish thorough performance specifications for this laboratory-developed assay. The *lytA* assay demonstrates excellent linearity across the entire linear range. The assay also demonstrates a high level of precision and repeatability, with acceptable levels of random error. Further, the sensitivity and specificity of the assay are both very high, so the test identifies pneumococcus in extremely low concentrations, while at the same time not detecting similar organisms. Finally, we find that the *lytA* assay is in 100% agreement with *S. pneumoniae* gold-standard reference identification methods.

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I would also like to thank my colleagues in the Bacterial Pathogens Research Unit here at Rollins. Many of the laboratory procedures required for the methods of this study were beyond my skill set and I counted on the expertise and hard work of these individuals. I would especially like to thank Dr. Fuminori Sakai and Virginia Stringer who conducted many of the quantitative PCR assays needed for this study. Without them, there would have been little to write about.

- David

Introduction

Pneumonia is the single leading cause of child death worldwide, killing more than 1 million children under the age of five each year. Even in the United States, despite advances in care, pneumonia is still the seventh leading cause of death, posing a serious threat to children and also the elderly. The most common cause of pneumonia is the bacteria *Streptococcus pneumoniae*, also called pneumococcus. Cases of community acquired bacterial pneumonia are often severe, especially in young children. Carriage of the pneumococcus is a prerequisite for infection, and carriage rates in children are extremely high.

Diagnosing a case of pneumococcal pneumonia can be very difficult due to the inherent drawbacks of the current gold standard methods based on bacterial culture of sputum and blood. These traditional methods have both low specificity and even lower sensitivity and it can take several days to obtain a laboratory confirmed diagnosis. There is a need for the development of new tests which take advantage of modern advances in microbiological technology. Recently several new PCR procedures have been developed to identify specific genes in the pneumococcus.

A good example of this is the *lytA* assay, which was developed in 2007 by Carvalho et al. at the Centers for Disease Control and Prevention (Carvalho Mda et al., 2007). This assay has been used in carriage studies, disease surveillance, vaccine guidance and maybe even clinical diagnosis of pneumococcal disease. First however, the assay must be validated for use in diagnostic and clinical laboratories. This report documents the process and results of our validation studies for the *lytA* qPCR assay.

As part of a phase III clinical trial being conducted by Cempra, Inc., Dr. Jorge Vidal's laboratory at Emory University set out to validate the *lytA* assay. This particular assay had never been used as part of a clinical trial previously, so the US Food and Drug Administration required

the assay to be formally validated prior to the submission of any trial data acquired using the assay. In order to validate this laboratory-developed test, we conducted a thorough series of validation experiments, planned in accordance with the regulations laid out in the Clinical Laboratory Improvement Amendments (CLIA). We based our study methodology off of guidelines published by the Clinical and Laboratory Standards Institute (CLSI) and International Organization for Standardization (ISO).

Establishing the performance specifications for the *lytA* assay will allow future research to be confidant in the test's capabilities for producing accurate results which have high levels of both sensitivity and specificity. I believe that the *lytA* assay will become a more commonly used tool in clinical studies and perhaps even be adapted into a clinical test for use in diagnosing cases of pneumococcal pneumonia.

Abbreviation Listing

Abbreviation	Definition
ARI	Acute respiratory infection
ATCC	American Type Culture Collection
CAP	Community acquired pneumonia
CABP	Community acquired bacterial pneumonia
CDC	Centers for Disease Control and Prevention
CLIA	Clinical Laboratory Improvement Amendments
CLSI	Clinical and Laboratory Standards Institute
CFU	Colony forming units
CI	Confidence interval
Cq	Quantitation cycle
DNA	Deoxyribonucleic acid
FDA	US Food and Drug Administration
ISO	International Organization for Standardization
LOD	Limit of detection
NEC	Negative extraction control
NP	nasopharyngeal
NPV	Negative predictive value
NTC	No template control
PPV	Positive predictive value
qPCR	Quantitative real time polymerase chain reaction
STGG	Skim milk, tryptone, glucose, glycerin (sample media)

Literature Review

Epidemiology of pneumonia

Acute respiratory infections (ARI) are the leading cause of childhood disease worldwide. A child under five years of age will experience approximately three to six episodes of ARIs annually, regardless of where they live or their economic status (Eric A. F. Simoes, 2006). These disease episodes can be very serious, and sometimes in severe cases, even fatal. Approximately 1.9 million (95% CI 1.6-2.2 million) children died from ARIs in 2000, 70% of them in Africa and Southeast Asia (Eric A. F. Simoes, 2006). Pneumonia, one of the most common ARIs, is the single leading cause of child death worldwide, accounting for nearly 1 million deaths per year in children under 5 (Austrian, 1999). This is more than HIV/AIDS, malaria, and measles combined (T. Wardlaw, Salama, Johansson, & Mason, 2006). Only diarrheal disease approaches the number of deaths caused by pneumonia. Even in the United States, despite advances in care, pneumonia is still the seventh leading cause of death, posing a serious threat to children and the elderly. At the same time however, the impact of pneumonia on children, much like the impact of diarrheal disease, is often overlooked by mainstream media and even by the medical community. UNICEF and WHO have called pneumonia "The Forgotten Killer of Children" (Tessa Wardlaw, Johansson, & Hodge, 2006).

Physiopathology of pneumonia

Pneumonia is an ARI which affects the alveoli of the lungs. The condition, mainly caused by a viral or bacterial infection, develops when the alveoli begin to fill with pus and liquid, making

it difficult or even painful to breath ("Pneumonia Fact Sheet (#331)," 2015). Clinical manifestation of pneumonia include sudden onset high fever, pleural pain, dyspnoea, tachypnoea, and cough with associated “rusty” sputum (*Control of Communicable Diseases Manual*, 2004). In young children, fever, vomiting, and convulsions are often the initial manifestation. Pneumonia is spread several different ways, depending on the types of pathogens associated. Viral and bacterial pneumonias are often caused by inhalation of the pathogen. The pathogen is spread via air-borne droplets from a cough or sneeze of another infected individual, or a pathogen that normally resides in the nose and/or throat is aspirated into the lungs ("Pneumonia Fact Sheet (#331)," 2015). This second means of spread is of great importance for the bacterial pathogens that cause pneumonia, such as *Streptococcus pneumoniae*.

Pneumococcal pneumonia

Streptococcus pneumoniae (pneumococcus) is a gram-positive encapsulated coccus bacteria which is the main causative agent for community acquired pneumonia (*Control of Communicable Diseases Manual*, 2004). Pneumococcus commonly colonizes the human nasopharynx, but can be found throughout the respiratory tract of healthy individuals. Colonization of the nasopharynx by pneumococcus is most prevalent in children under two years old, but can be found in adults. Studies have shown that as many as 70% of all children asymptomatically carry pneumococcus in the nasopharynx (Simell et al., 2012). Aside from pneumonia, pneumococcus is also one of the main causes of otitis media, sinusitis, meningitis and bacteremia ("Pneumococcal Disease," 2015).

Although pneumococcus is a well-documented and potentially lethal pathogen, it is a normal member of the upper respiratory tract. This means that, while pneumococcus can cause

disease, many people are simply asymptomatic carriers of those bacteria (Song, Eun, & Nahm, 2013). The human nasopharynx and orpharynx serves as the reservoirs of *S. pneumoniae* in the body (Bogaert, de Groot, & Hermans, 2004). The nasopharynx of healthy humans for example, can be colonized by pneumococci as early as infancy, when 30-60% of infants are found to carry the bacteria. Carriage rates decrease with age, finally settling at less than 10% in adults, but they can be as low as 2% in adults from industrialized countries (Almeida et al., 2014). Carriage of pneumococcus is extremely important because it is a prerequisite for pneumococcal disease including pneumonia (Simell et al., 2012).

Vaccination with pneumococcal vaccines

Available vaccines for the prevention of pneumococcal infection include the pneumococcal conjugate vaccines PCV7, PCV10, and in most developed countries, PCV13 and the pneumococcal polysaccharide vaccine PPSV23. PCV7, the first of the conjugate vaccines for pneumococcus, was first adopted in the U.S. in 2001 and widely in Europe by 2006. PCV13 largely replaced PCV7 and PCV10 in developed countries after 2010 (Weil-Olivier, van der Linden, de Schutter, Dagan, & Mantovani, 2012). PCV13 is designed to protect against 13 pneumococcal bacterial serotypes. This vaccine is primarily for children and all adults over 65 years old, or adults with specific health problems (CDC, 2015). PPSV23 is designed to protect against 23 pneumococcal bacteria serotypes. It is recommended for adults 65 years of age and older who have already received the PCV13 vaccine, immune suppressed adults, and adults who smoke or have asthma.

In 2012, 70% of high income countries included a PCV vaccine in their national vaccination schedule (Fitzwater, Chandran, Santosham, & Johnson, 2012). Prior to 2008 no

developing countries included PCVs in their schedules, however since then more than 40 low income countries have taken steps to universally distributing the vaccines. Ever since the introduction of PCV7 in the United States and Europe, rates of pneumococcal disease have steadily fallen, particularly among children. In the United States, hospitalizations due to pneumococcal disease among children under the age of five dropped from 82.7 to 2.2 hospitalizations per 100,000 children over the three years following the introduction of PCV7 (Simonsen et al., 2011). A cost-effectiveness study of pneumococcal vaccination projected that the vaccines will prevent 262,000 deaths per year (7%) in children aged 3–29 months in the 72 developing countries included in the study. The study also predicted that 100% vaccine coverage in children could prevent 407,000 deaths per year (Sinha, Levine, Knoll, Muhib, & Lieu, 2007).

Community acquired bacterial pneumonia (CABP) and Clinical Diagnosis

While some cases of bacterial pneumonia originate in a healthcare setting, the majority are acquired in the community setting. Community acquired bacterial pneumonia (CABP) is simply a case of confirmed pneumonia which occurs in a person that has not been exposed to a healthcare system. When a case of CABP develops, early and accurate diagnosis is associated with both improved patient outcomes and reduced cost of care (Houck, Bratzler, Nsa, Ma, & Bartlett, 2004). Pneumonia can be caused by a variety of infectious agents, and therefore treatment strategies can vary drastically. Rapid and accurate diagnosis leads to more targeted and effective treatments earlier in the disease's pathophysiology. This also reduces unnecessary or inappropriate treatments, which waste both time and resources. Actually isolating pneumococcus in a normally sterile part of the body provides a conclusive positive diagnosis, however this is very rare. Obtaining quality samples without using excessively invasive procedures is difficult (Werno &

Murdoch, 2008). In a clinical setting, practitioners often rely on other means to obtain a diagnosis when CABP is suspected.

Diagnosing cases of community acquired pneumonia (bacterial or otherwise) hinges primarily on the presence of specific clinical features including fever, cough, the production of sputum, and chest pain. These findings must be supported by radiographic imagery of the chest and lungs (Mandell et al., 2007). Physicians may also conduct a physical examination in order to detect abnormal or bronchial breath sounds. While physical examination may be an important component of the overall evaluation, they are significantly less sensitive and specific than the radiograph of the lungs (Wipf, Lipsky, Hirschmann, & et al., 1999). In the clinical setting, a chest radiograph is used to differentiate suspected CAP from many of the other common causes of fever and raspy cough, such as acute bronchitis (Mandell et al., 2007). When a case of community acquired pneumonia is diagnosed, it is important to confirm the specific infectious agent. This aids in guiding the treatment plan as well as accumulating epidemiological data for advancing collective understanding of CABP.

Laboratory diagnostics for the identification of pneumococcal pneumonia

Microbiological diagnostic testing for pneumonia is conducted in a diagnostic lab. This is a crucial step, especially when attempting to confirm whether a case of pneumonia is caused by *S. pneumoniae*. The current gold standard for diagnosis of pneumococcal pneumonia in a laboratory setting requires multiple procedures. CABP must first be suspected due to the associated symptoms listed above. Besides radiographic and clinical evidence, the definitive diagnosis of pneumococcal pneumonia include at least one of the following (Werno & Murdoch, 2008):

1. Isolation of the pneumococcus from a normally sterile body fluid, including blood and pleural fluid
2. Isolation of the pneumococcus from sputum samples of microbiological quality, ie ≥25 polymorphonuclear leukocytes and ≤10 epithelial cells (Werno & Murdoch, 2008)
3. Positive urinary antigen test

Cultures and identification of *S. pneumoniae*

To isolate the bacteria from body tissue, lung tissue needs to be obtained by biopsy or bronchoscopy or transtracheal aspiration (Song et al., 2013). These approaches are highly invasive and impractical in a clinical setting however, so the majority of bacteriological confirmation is actually obtained from more easily acquired fluid samples such as peripheral sputum, blood samples, and pleural fluids (Song et al., 2013). The fluids are refined and the samples are incubated overnight at 35°C with 5% CO2 on 5% sheep blood agar or chocolate agar plates. *S. pneumoniae* colonies generally appear as small, gray, and mucoid mounds and they are surrounded by a zone of discolored agar due to α-hemolysis (Werno & Murdoch, 2008). A Gram stain is used to determine if the bacteria being investigated is a gram-positive diplococci. A catalase test is performed to distinguish streptococci species from staphylococci species, i.e., pneumococci are negative.

In order to distinguish pneumococcus from other streptococci species, two separate tests must be conducted. The first test is for optochin susceptibility. Optochin (ethylhydrocupreine hydrochloride) is a chemical which is toxic to *S. pneumoniae*, but not toxic towards most other species of streptococci (Chandler, Reisner, Woods, & Jafri, 2000). For this test, a disk impregnated with optochin is placed on a blood agar plate which has been freshly streaked with a colony from

the original plate. The plate is allowed to incubate overnight under standard conditions. Reading the optochin test is very simple. Using a ruler or calipers, the zone of inhibition around the disk is measured. If this zone is greater than or equal to 14 mm, then the bacteria can be presumptively identified as *S. pneumoniae* (Dana Castillo, 2011). If the zone is smaller an identification cannot be made. The sample could be either another species of streptococci or a pneumococcus strain which is resistant to optochin. If this is the case, then a bile solubility test must be conducted.

The bile solubility test is used to further distinguish *S. pneumoniae* from all other alpha-hemolytic streptococci species. Sodium deoxycholate, the sodium salt of the secondary bile acid deoxycholic acid, is used as a biological detergent in this procedure. *S. pneumoniae* is bile-soluble (meaning that bile acids will lyse the cells), whereas all of the other alpha-hemolytic streptococci species are bile-resistant. 2% Sodium deoxycholate diluted in sterile water will lyse the cell walls of pneumococci. The turbidity of the solution after 10 min and 2 h determines the tests results. A clear solution which is associated with a turbid control (saline added to water instead of bile salt) will indicate a positive result (Dana Castillo, 2011). Further gold standard tests for determining serotype include the Quellung reaction or latex agglutination (Satzke et al., 2013). In the Quellung reaction, specific antibodies were added to a suspension of the bacteria on a glass slide. The cells were then viewed under a microscope. We looked for capsular swelling which demarcated a positive identification (Habib, Porter, & Satzke, 2014).

While the isolation of *S. pneumoniae* from sterile sites or sputum, as mentioned above, is currently the accepted method for diagnosis of pneumococcal infection, they have some significant limitations. The primary limitations include low sensitivity. Different studies have reported a wide range of values for diagnostic sensitivity of culture techniques, from 29 to 94% (Song et al., 2013). This is due primarily to the quality of sputum samples obtained for microbiological diagnostics. A

2000 study found that sputum culture identified pneumococci only 44% of the time in patients with bacteremic pneumococcal pneumonia (Musher et al., 2000). On top of this sputum culture can also produce false positives when the sample is contaminated with colonizing bacteria from the nasopharynx. Even when the traditional culture methods work properly, they can take up to several days to complete. Urinary antigen tests have shown some promise as an alternative to culture. Studies of the efficacy of the Binax NOW urinary antigen test conducted with adults showed reasonable sensitivity and very high specificity (Murdoch et al., 2001). Other studies, however, have found that the Binax NOW test was unable to distinguish between carriage and infection in children, while being no more sensitive than traditional diagnostic methods (Dowell, Garman, Liu, Levine, & Yang, 2001). The detection ability of the Binax NOW antigen test is also limited to only the 13 PCV13 serotypes.

Polymerase chain reaction

The limitations of the accepted methods emphasize the need for modern PCR techniques. PCR assays for the identification of *S. pneumoniae* have shown increased sensitivity and specificity in a number of studies. In 2007, Carvalho et al. developed a novel real-time PCR which targeted the *lytA* gene, an ~80 bp fragment unique to *S. pneumoniae* (Carvalho Mda et al., 2007). This new assay demonstrated high specificity and sensitivity. The high specificity of the *lytA* assay was extremely important because it means that the assay can be used on samples collected from non-sterile sites, such as the nasopharynx.

As part of a phase III clinical trial being conducted by Cempra, Inc., Dr. Jorge Vidal's laboratory at Emory University set out to validate the *lytA* assay. For this clinical trial the *lytA* assay was used to identify the presence and concentration of pneumococcus in the nasopharynx of

trial participants. As this particular assay had never been used as part of a clinical trial previously, The US Food and Drug Administration required the assay to be formally validated prior to the submission of any trial data acquired using the assay. In order to validate this laboratory-developed test, we conducted a thorough series of validation experiments. These experiments were planned in accordance with the regulations laid out in the Clinical Laboratory Improvement Amendments (CLIA) of 1988. The CLIA federal regulatory standards (Public Law 100-578) were updated to their current state in 2003 (Burd, 2010). While CLIA does list the performance specifications that must be established when validating a laboratory-developed test, it does not specify the methodology or specific data analysis tools which should be used to acquire performance specifications. As such, we based our study methodology off of guidelines published by the Clinical and Laboratory Standards Institute (CLSI) and International Organization for Standardization (ISO) as summarized in the report *Validation of Laboratory-Developed Molecular Assays for Infectious Disease* by Dr. Eileen M. Burd (Burd, 2010).

Methods

The *lytA* Assay

The *lytA* assay which was the subject of the validation study was conducted in the following manner:

DNA extraction from nasopharyngeal samples.

All DNA extractions which were conducted as part of this validation study occured in an access-restricted laboratory in the Claudia Nance Rollins Building at the Rollins School of Public

Health, Emory University. Extractions were performed within a sterile biological safety cabinet. Nasopharyngeal swabs which had been stored at -80°C were thawed at room temperature and then vortexed for 15 seconds. 200 µl of this sample was then added to 100 µl of TE (Tris-EDTA) buffer which contained 75 U/ml of mutanolysin and 0.04 g/ml lysozyme and then incubated in a 37°C water bath for 1 h. The subsequent steps are outlined as indicated in the QIAGEN DNA Mini protocol booklet (Qiagen, 2012). Briefly, 20 µl of proteinase K and 200 µl of buffer AL were added to the samples and these samples were vortexed for 5 seconds. The tubes were incubated at 56°C in water bath for 30 min and then briefly centrifuged to remove drops from inside of the lid. The tubes were treated with 260 µl of 96% molecular biology grade ethanol and the mixture was vortexed for 15 s. This mixture was transferred to a QIAamp Spin column and centrifuged at 6000 x g for 1 min. Eluted liquid was discarded into a biohazard container and the Qiagen spin column was installed in a new collection tube. The column was washed with 500 µl of buffer AW1 followed by 500 µl of buffer AW2. After washing, the column was placed into a sterile 1.5 ml eppendorf tube, along with 100 µl of elution buffer (AE), and incubated 5 min at room temperature.

To elute the DNA, the tubes were centrifuged at 6000 x *g* for 1 min and immediately stored at -80°C. DNA from the *S. pneumoniae* reference strain TIGR4 was also extracted from overnight cultures in Todd-Hewitt broth supplemented with 0.5% of yeast extract (THY) as mentioned above. DNA concentrations were obtained by the Nanodrop method (Nanodrop Technologies, Wilmington, DE).

DNA extraction of *S. pneumoniae* reference strain TIGR4

TIGR4 DNA was used in a range of concentrations as a positive control/ standard for the *lytA* assay. TIGR4 was inoculated on a blood plate agar and incubated overnight at 37°C in a 5%

CO_2 atmosphere. Two heat blocks were set at 56°C and 70°C. A bacterial suspension was made with 200 µl buffer ATL in a 1.5 ml eppendorf tube with 20µL proteinase K. This suspension was mixed by pulse-vortexing for 15 s and incubated at 56°C in a water bath for 1 h. The suspension was occasionally vortexed during the incubation period to homogenize the sample. At the end, 4 µl RNase solution (100 mg/ml) was added and again mixed by pulse-vortexing for 15 s after which it was incubated for 2 m at room temperature. Next, 200 µl of buffer AL was added to the suspension, mixed by pulse-vortexing for 15 s, and incubated at 70°C for 10 min. An aliquot of 200 µL of ethanol (96-100%) was added to the sample, mixed by pulse-vortexing for 15 s and the mixture was applied to the QIAamp Mini spin column without wetting the rim. The sample was centrifuged at 6000 x g (8000 rpm) for 1 min and the QIAamp Mini spin column was transferred into a clean 2 ml collection tube. The column was added with 500 µl buffer AW1 and centrifuged at 6000 x g (8000 rpm) for 1 min after which the column was again transferred to a clean 2 ml collection tube. 500 µL buffer AW2 was then added to the column and centrifuged at full speed (20000 x g; 14000 rpm) for 3 min. The column was then transferred to a sterile 2 ml eppendorf tube and 200 µl of elution buffer AE was added. Finally, the column was incubated at room temperature for 5 min, then centrifuged at 6000 x g (8000rpm) for 1 min and the eluted DNA was stored immediately at -80C until use.

Preparation of standards for quantitative polymerase chain reaction

Purified DNA was diluted to give a final concentration in each reaction tube corresponding to 1 ng, 100 pg, 10 pg, 1 pg, 0.1 pg and 0.05 pg. These standards represented the following copy numbers (genome equivalents): 4.29E+05, 4.29E+04, 4.29E+03, 4.29E+02, 4.29E+01 and 2.14E+01, respectively. These genome equivalents were calculated using the formula shown

below. The genome size of *S. pneumoniae* TIGR4 is ~2,160,842 base pairs (bp). Using Avogadro's number, 6.022x1023 molecules/mole, the number of molecules of the template per gram can be calculated as follow:

*1.0ng DNA= 6.022*10^23/ (genome length*1*10^9*650) copy=428754 copy*

Quantitative PCR (qPCR)

A qPCR assay that targets the autolysin *lytA* gene, present in all *S. pneumoniae* strains, was performed. The qPCR assay utilized the following primers and probe: *lytA*-CDC forward 5-ACG CAA TCT AGC AGA TGA AGC A-3; *lytA*-CDC reverse 5-TCG TGC GTT TTA ATT CCA GCT-3; *lytA*-CDC probe 5-FAM-TGC CGA AAA CGC TTG ATA CAG GG AG-3-BHQ1 (Carvalho Mda et al., 2007) . Quantitative PCR reactions were carried out in a final 25 µl volume and performed using the Invitrogen Platinum qPCR Master Mix-UDG (Invitrogen), with 2.5 µl of DNA template and 200 nM of each primer and probe.

S. pneumoniae encodes only one copy of the *lytA* gene (our target), so the number of copies detected in the samples were equivalent to the number of bacteria present [colony forming units/ml (CFU/ml)]. All qPCR reactions were run in a CFX96 real time system (BioRad, Hercules, CA) with the following cycling parameters: 95°C for 2 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Negative samples had no Cq value. CFU was finally calculated using the BioRad CFX manager software (BioRad, Hercules, CA).

Assay Quality Assurance

Quality controls

All runs of the *lytA* assay contained both positive and negative controls. Sterile DNA-grade water served as the negative extraction control (NEC). It was added to an empty sample DNA extraction column during the DNA prep stage of the protocol, in order to verify that there was no cross contamination during DNA extraction. The NEC was added as a template in the read software and was run in duplicate in two wells.

Sterile DNA-free PBS served as a PCR negative control (no template control [NTC]). It was added as template in the run software and was run in duplicate or triplicate. The NTC was used to evaluate whether or not contamination may have occurred during the preparation of the qPCR master mix.

The positive controls for the *lytA* assay were the TIGR4 DNA standards generated from the *S. pneumoniae* reference strain ATCC BAA-334 (TIGR4) and then quantified by NanoDrop™. The seven standards, their concentrations, the conversions to *lytA* gene copies, and *lytA* gene copies/mL (equivalent to CFU/mL) are outlined in Table 3.

Acceptance criteria

There are several essential acceptance criteria which all runs needed to pass in order for their results to be recorded. If these acceptance criteria were not met, the assay was rerun along and any other corrective actions deemed necessary were applied. The acceptance criteria are as follows.

1. Efficiency ratings output by the BioRad CFX manager software must fall within the permitted range (90% < x < 110%). If the efficiency was outside of the accepted range, then all controls and samples in the plate were repeated in the qPCR assay.
2. The negative extraction control (NEC) must be negative. If the NEC was positive (Cq ≠ 0), samples within that extraction set were repeated from the extraction step and retested by qPCR.
3. The no template control (NTC) must be negative. If the NTC was positive (Cq ≠ 0), the master mix was prepared again from scratch and the qPCR reaction was be re-run.

Recording of Data/Observations

Quantification data (CFU/ml) for each run was calculated using the BioRad CFX manager software and results were stored on a Dell desktop computer with restricted access to approved users. All data critical to the study was backed up to the RSPH Server in access restricted folders. The server is backed up and secured by the RSPH Information Technology Department.

Experiments

The primary goal when validating a laboratory-developed test is to establish the test's performance characteristics. These characteristics include accuracy, precision, reportable range, analytical sensitivity, and analytical specificity. In order to establish the performance characteristics of the *lytA* assay a series of experiments were conducted.

Reportable Range

The reportable range is the span of test result values which can establish the accuracy of the instrument or test system measurement response (Burd, 2010). Reportable range is also commonly referred to as a test's "linear range". The reportable range is capped off on both ends by the lower limit of quantification (LLOQ) and the upper limit of quantification (ULOQ). A linearity experiment was conducted to determine the reportable range of the *lytA* assay. Purified *S. pneumoniae* TIGR4 genomic DNA standards (see Table 3) were run in single or duplicate reactions across eleven testing days. In total, 22 replicates of each of the 7 standards were tested. A line of best fit for the assay was then calculated.

Analytical Sensitivity

Analytic sensitivity is the ability of an assay to detect low concentrations of the given substance or biological specimen (Burd, 2010). The limit of detection (LOD) is the quantitative measure of an assay's analytic sensitivity, and is specifically defined as the lowest concentration with a detection rate ≥ 95%. We conducted several preliminary studies utilizing the *lytA* assay prior to validation which indicated a limit of detection (LOD) of ~2 copies/reaction for the *S. pneumoniae* TIGR4 reference strain. Therefore, 2 copies/reaction was the first LOD tested in this validation study ("predicted LOD"). In order to determine the actual analytical sensitivity (LOD) of the *lytA* qPCR assay, 5 different ATCC strains of *S. pneumoniae* were tested. Each of these strains was diluted to 3 concentrations near the predicted LOD of 2 copies/reaction: 1.07, 2.14 and 4.28 copies/reaction. The LOD for each of the three *S. pneumoniae* strains was determined, and the overall LOD of the assay was identified as the highest LOD of the three different *S. pneumoniae* strains tested.

Precision

A precision study was performed in order to determine how well the *lytA* qPCR test can reproduce consistent results when applied to standard samples of known concentrations (Burd, 2010). For this study the assay was conducted repeatedly, using five ATCC *S. pneumoniae* strains at 4 known concentrations. Each of the 20 different strain-concentrations was run in duplicate, 1-2 times per day, for 20 testing days. In total, 78 replicates were collected at each of the 20 strain-concentrations. The concentrations tested were chosen because they spanned the measuring range of the assay (4, 5, 500, and 4.0E+05 copies/reaction).

Standard deviations and 95% confidence intervals (CIs) of the quantitation cycle (Cq) values were collected for each strain-concentration (n=20) as well as all strains combined at each concentration (n=4).

Analytical Specificity

In order to determine the ability of the *lytA* qPCR assay to detect only the intended target, we next conducted a specificity study. The goal of the study was to determine if the assay consistently detected the single copy streptococcal *lytA* gene, while at the same time not exhibiting any cross-reactivity with unintended targets (Burd, 2010). The specificity panel of unintended targets included the nucleic acids of the following: organisms with similar genetic structure to *S. pneumoniae*, normal flora organisms that may be present in nasopharyngeal swabs, and organisms that cause similar diseases to pneumonia. See Table 1 for the full list of species in the specificity panel.

For the specificity study, all nucleic acids for each organism listed in Table 1 were isolated using the QIAamp DNA Mini Kit (Qiagen), quantified by NanoDrop™ (Thermo Scientific) and tested in the *lytA* qPCR assay at 100 pg DNA/reaction, in triplicate (This concentration corresponds to Standard concentration #2 for TIGR4). TIGR4 was included in the specificity study as a positive control.

Accuracy

Next, an accuracy study was conducted to determine the degree of "closeness" between the results obtained by the *lytA* qPCR assay and those results obtained utilizing the gold standard reference identification method (Burd, 2010). 40 well characterized isolates of *S. pneumoniae*, which were sourced from The Centers for Disease Control and Prevention (CDC) and American Type Culture Collection (ATCC), were used in the study. All of the strains included in the accuracy panel were first verified by the conventional culture, then by real-time PCR assays and Quellung Reaction. The conventional culture methods to identify *S. pneumoniae* included: observations on colony morphology and hemolysis on blood agar plates, the optochin sensitivity assay, and the bile salt solubility assay. Two different real time PCR assays, specific to *S. pneumoniae*, were used. The first targeted the pneumolysin gene (*ply*) (Carvalho Mda et al., 2007)and the second targeted a capsular polysaccharide biosynthesis gene (*cpsA*) (Park, Lee, & Kim, 2010). See Table 2 for the full list of *S. pneumoniae* isolates in the accuracy panel.

All *S. pneumoniae* isolates for this study were first grown on agar plates. A single loopful of each isolate was then spiked into STGG sample media. The simulated *S. pneumoniae*-positive specimens were subjected to DNA extraction utilizing the QIAamp DNA Mini Kit (Qiagen), and then *lytA* qPCR. Simulated samples were extracted and each nucleic acid sample (40 total samples)

was tested in triplicate along with a TIGR4 standard curve (positive control) and negative PCR control (no template control, NTC) in a standard *lytA* assay.

Results

Reportable Range (Linearity)

In total, 22 replicates of the 7 TIGR4 standards were tested over the span of 11 separate testing days. Using the linear regression equation for each data set to obtain the "measured value", the qPCR Cq values were then converted to log10 copies per qPCR reaction. The assigned value, given in log10 copies/mL of each standard, are presented in Table 3. The assigned values are also plotted against the measured value for all replicates (Figure 1).

Linear regression conducted on this data set provided the equation y = 1x – 2E-05 (R2 of 0.9941). This equation demonstrates the strong linear relationship across the full range tested, from 2.14 copies/reaction to 429,000 copies/reaction. This range corresponds with a concentration of 428 to 8.58E+07 CFU/mL in the original sample.

Analytical Sensitivity (Limit of Detection)

Sensitivity studies were performed to determine the LOD of the assay (lowest analyte concentration that can be reliably detected in ≥ 95% of specimens). Five different ATCC strains of *S. pneumoniae* were evaluated at 3 concentrations near a predicted LOD of 2 copies/reaction.

Detection rates for each strain/concentration are shown in Table 4, and LODs for each strain are highlighted. The LODs are 2.14 copies/reaction for the BAA-334 (TIGR4) and ATCC 49619 strains, and 4.28 copies/reaction for the BAA-255, ATCC 700676 and ATCC 33400 strains. Therefore, the overall LOD for *S. pneumoniae* detection by the *lytA* qPCR assay based on this data set is 4.28 copies/reaction. This corresponds to approximately 856 CFU/mL in a primary clinical specimen.

This LOD is supported by data in the precision study where the concentration of 4 copies/reaction demonstrated an overall detection rate of 95.1% across these five ATCC *S. pneumoniae* strains, tested in duplicate, 1-2 times per day on each of 20 testing days. An LOD of 4 copies/reaction corresponds to 800 CFU/mL in a primary clinical specimen. Taking both LOD and precision study data together, the LOD of the assay is 4 copies/reaction.

Precision

The precision study was performed using 5 ATCC *S. pneumoniae* strains at 4 concentrations. Each of the strains, at each of the 4 concentrations, was tested in duplicate, 1-2 times per day on each of 20 testing days for a total of 390 replicates at each concentration (78 replicates at each concentration for each of 5 *S. pneumoniae* strains). The concentrations chosen for testing spanned the measuring range of the test system (from near the LOD of 4 copies/reaction up to 4.0E+05 copies/reaction). See Table 5 for complete results for each of the 5 strains tested.

The detection rate across all five strains at the concentration of 4 copies/reaction was 95.1%, further supporting the previously conducted LOD determination studies which demonstrated that the LOD of the *lytA* assay was 4.28 copies/reaction. Standard deviations across the five strains tested were 0.385 and 0.389 at the high and mid-level concentration, respectively,

and 1.008 and 1.057 at the concentration just above the LOD and at the LOD, respectively. For the averaged results of all the strains organize by concentration, see Table 6.

Analytical Specificity

A panel of select organisms (n=29) were evaluated in the *lytA* qPCR assay. The organisms chosen for the panel included a list of pathogens which are commonly found in sputum or nasopharyngeal samples including normal flora organisms, and organisms that can cause similar disease including other streptococcal species, (See Table 1). The nucleic acid purified from each strain was tested in triplicate at 100 pg DNA/reaction using the standard *lytA* assay. The chosen concentration corresponds with TIGR4 Standard #2 (4.3E+04 copies/reaction), which is at the higher end of the standard curve.

In total, all replicates of the 29 organisms tested were not detected by the *lytA* qPCR assay. The target control strain *S. pneumoniae* ATCC BAA-334 (TIGR4) was positive in all tests conducted (mean Cq = 23.4). This study demonstrated 100% specificity for all of the organisms tested. The assay has displayed no instance of cross reactivity.

Accuracy

The accuracy study was conducted with 40 well characterized clinical isolates of *S. pneumoniae*, all of which were sourced from either Emory University or the Centers for Disease Control and Prevention (Table 7). All of the isolates listed in the panel were verified by the following: colony morphology; hemolysis on blood agar plates; bile salt solubility; optochin sensitivity; ply real time PCR; cpsA real time PCR; and, Quellung Reaction.

These simulated *S. pneumoniae*-positive clinical specimens were subjected to DNA extraction and *lytA* qPCR per the same protocol used for testing clinical specimens. All of the 40 accuracy panel specimens were tested in triplicate. The *lytA* assay found all of the 40 *S. pneumoniae*-spiked specimens to be positive, indicating that the assay was in 100% agreement with *S. pneumoniae* gold-standard reference identification methods.

Discussion

In this study we set out to validate the *lytA* assay, which was developed in 2007 by Carvalho et al. at the Centers for Disease Control and Prevention (Carvalho Mda et al., 2007). Prior to this study, the assay has not yet been fully validated for use in diagnostic and clinical laboratory studies. The experiments conducted in this study display the efficacy of the *lytA* assay and allow us to establish thorough performance specifications for this laboratory-developed assay, including reportable range, analytical sensitivity, precision, analytical specificity, and accuracy. We find that the *lytA* assay demonstrates excellent linearity across the entire linear range. The assay also demonstrates a high level of precision and repeatability, which means that, when the procedure is conducted properly, there is little chance of random error. Further, the sensitivity and specificity of the assay are both very high, so the test identifies pneumococcus in extremely low concentrations, while at the same time not detecting similar organisms, including other streptococcal species and other bacteria common in the respiratory tract. Finally, we find that the *lytA* assay is in 100% agreement with *S. pneumoniae* gold-standard reference identification methods.

The limit of detection (LOD) of the *lytA* qPCR assay is 2.14 copies/reaction for *S. pneumoniae* strains BAA-334 (TIGR4) and ATCC 49619, and 4.28 copies/reaction for *S. pneumoniae* strains BAA-255, ATCC 700676 and ATCC 33400. Precision testing across these 5 strains demonstrated a 95.1% detection rate at 4 copies/reaction. Therefore the overall LOD of the assay is 4 copies/reaction. Further, the *lytA* qPCR assay is linear across the range tested, from 2.14 to 429,000 copies/reaction, and therefore demonstrates acceptable quantification down to the LOD of the assay. This LOD of 4 copies/reaction is equal to a bacterial load of 800 CFU/mL in a primary clinical specimen.

The *lytA* qPCR assay demonstrates high reproducibility across test setups, over time, across strains and over the measuring range of the assay, which will allow for testing clinical isolates with high reliability. The results obtained in the precision study are very similar for all of the strains tested, demonstrating the consistency of the *lytA* qPCR assay across several different *S. pneumoniae* strains. The results of this study display very low instance of random analytical error. Despite changes in strain and concentrations, and even over long spans of time, the *lytA* assay shows very high levels of reproducibility.

The *lytA* qPCR assay demonstrates amplification of all 40 *S. pneumoniae* reference strains from simulated clinical specimens, but does not amplify any of the other non-pneumococcal streptococcus or non-streptococcal organisms tested (n=29). These results, along with previously published reports, demonstrate excellent accuracy (100%) and analytical specificity (100%) for testing clinical isolates.

These results support previous studies that also demonstrated 100% analytical specificity for the *lytA* qPCR assay. Carvalho 2007 conducted a specificity study using nucleic acids extracted from 67 *S. pneumoniae* isolates and 104 non-pneumococcal isolates (at concentrations of 5 ng/µL,

or 12.5 ng/qPCR reaction) (Carvalho Mda et al., 2007). The non-pneumococcal isolates represented several genera of gram-positive and gram-negative bacteria, a portion of which naturally inhabit the oral cavity. They demonstrated *lytA* amplification in all 67 *S. pneumoniae* samples and no *lytA* amplification of all 104 non-streptococcal samples.

Conclusions and Recommendations

The limitations of traditional culture-based methods for the diagnosis of *S. pneumoniae* have been well documented. Principle among these is the low sensitivity and specificity of such methods (Song et al., 2013). PCR has shown lots of promise in recent years for being an extremely valuable diagnostic tool. The ability to rapidly and accurately diagnose pneumococcal pneumonia could have many benefits including: improvements to our ability to provide appropriate therapy, improvements in vaccine effectiveness, and more accurate estimates the disease burden. The findings of this study show that qPCR which targets the *lytA* gene is both a rapid and accurate means of identifying the presence of pneumococcus and its density.

We present extensive performance specifications for the assay, in accordance with the regulations laid out in the Clinical Laboratory Improvement Amendments (CLIA). The study methodology is based off of guidelines published by the Clinical and Laboratory Standards Institute (CLSI) and International Organization for Standardization (ISO). The results of this study served as an appendix in a phase III clinical trial submitted to the US Food and Drug Administration. In that trial, the assay was used in a novel way to identify and quantify pneumococcus in the nasopharynx of study participants. The FDA required the assay to be

formally validated prior to the submission of any trial data which was acquired using the assay. The experiments conducted in this validation study allow us to establish thorough performance specifications for this laboratory-developed assay, including reportable range, analytical sensitivity, precision, analytical specificity, and accuracy.

First, we determined the limit of detection (LOD) of the *lytA* qPCR assay. We conducted a linearity experiment which showed that the overall LOD of the assay is 4 copies/reaction. Further, the *lytA* qPCR assay is linear across the range tested, from 4.28 to 429,000 copies/reaction, and thus demonstrates excellent linearity down to the LOD of the assay. The *lytA* qPCR assay demonstrated high reproducibility across test setups, over time, across strains and over the measuring range of the assay, which allows for testing clinical isolates with high reliability. The results of this study display very low instance of random analytical error. Overall, the *lytA* assay shows very high levels of repeatability.

The assay demonstrated amplification of all *S. pneumoniae* strains from simulated clinical specimens, but did not amplify any of the other non-pneumococcal streptococcus or non-streptococcal organisms tested. These results, along with previously published reports, demonstrate excellent accuracy (100%) and analytical specificity (100%).

Perhaps most importantly of all, the *lytA* assay is a rapid procedure. Whereas traditional methods for diagnosing pneumococcus can take up to several days, this assay can be completed in a single afternoon, while producing more accurate results. I believe that the most significant potential use for the validated *lytA* assay will be in pneumococcal surveillance studies. Many samples can be processed rapidly and accurately in a batch, in order to determine bacterial presence and load. The decrease in time required to conduct this test as well, as the increase in accuracy, over traditional methods could allow for more extensive and wide reaching active surveillance of

pneumococcal carriage. Additionally, in clinical trials involving pneumococcus this assay could be used to recruit more trial participants who would have been incorrectly screened out of the trial utilizing traditional gold standard methods, saving both time and limited funds.

The major limitation of this study is that the *lytA* assay has been validated for research purposes only. In order to use the *lytA* qPCR for clinical and diagnostic purposes (i.e. diagnose pneumococcal infection), the assay will have to undergo more stringent testing by the anticipated manufacturer of the test. This means that, while the test may be validated for the purposes of this clinical trial, it cannot be used as a new means of diagnosing Pneumococcal pneumonia in clinical patients. We believe however, that with further research and more extensive testing, the *lytA* assay could become an accepted diagnostic method for identifying pneumococcal infections. An extensive reference interval study would need to be conducted, as well as more extensive version of studies done here, in order for a manufacturer to commercially produce a diagnostic test which used the *lytA* gene as a target for diagnosing such types of disease.

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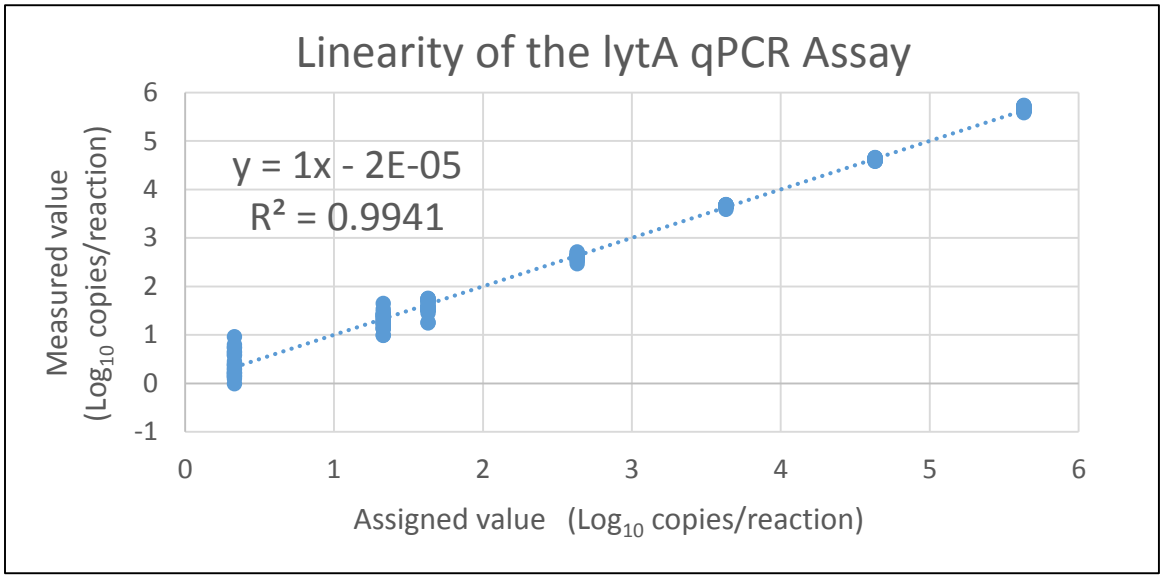
Appendices

Figures

Figure 1. Linearity of the *lytA* qPCR Assay



Tables

Table 1. *lytA* qPCR Specificity Panel Organisms

Positive Control Target Organism	**Source / ID**
S. pneumoniae (TIGR4)	ATCC BAA-334
Other Streptococcal Species (n = 19)	**Source / ID**
S. anginosus	ATCC 33397
S. australis	ATCC 700641
S. gordonii	ATCC 10558
S. infantis	ATCC 700779
S. intermedius	ATCC 27335
S. intestinalis	ATCC 43492
S. mitis	CDC SS-1303
S. mitis	ATCC 49456
S. mutans	ATCC 25175
S. oligofermentans	CDC SS-1725
S. oralis	ATCC 35037
S. parasanguinis	ATCC 15912
S. peroris	ATCC 700780
S. pseudopneumoniae	ATCC BAA-960
S. salivarius	ATCC 7073
S. sanguinis	ATCC 10556
S. sinensis	CDC SS-1726
S. sobrinus	ATCC 33478
S. vestibularis	ATCC 49124
Non-Streptococcal Organisms (n = 10)	**Source / ID**
Dolosigranulum pigrum	ATCC 51524
Haemophilus influenzae type A	CDC M6297
Haemophilus influenzae type B	CDC M5216
Haemophilus influenzae type C	CDC M6542
Haemophilus influenzae type D	CDC M6548
Haemophilus influenzae type E	CDC M9418
Moraxella catarrhalis	CDC 8121
Neisseria meningitidis	CDC 8201085
Staphylococcus aureus	ATCC 25923
Staphylococcus aureus Newman	ATCC 25904

Table 2 *lytA* qPCR Accuracy Panel Organisms: *S. pneumoniae* Strains

Type (n = 40)	Source
Serotype 1	CDC isolate 2012002345
Serotype 3	CDC isolate 2011212843
Serotype 4	CDC isolate 2011215139
Serotype 5	CDC isolate 2010220227
Serotype 6A	CDC isolate 2012002049
Serotype 6B	CDC isolate 2012203875
Serotype 6C	Emory isolate 20
Serotype 7C	Emory isolate 34
Serotype 7F	CDC isolate 2012214337
Serotype 8	Emory isolate 36
Serotype 9N	Emory isolate 86
Serotype 9V	Emory isolate 153
Serotype 9V	Emory isolate 381
Serotype 11A	Emory isolate 183
Serotype 11B	Emory isolate 229
Serotype 13	Emory isolate 711
Serotype 13	Emory isolate 716
Serotype 15C	Emory isolate 232
Serotype 16F	Emory isolate 257
Serotype 18C	Emory isolate 678
Serotype 18C	Emory isolate 218
Serotype 19A	CDC isolate 2012215778
Serotype 19F	CDC isolate 2012214924
Serotype 20	Emory isolate 336
Serotype 22F	Emory isolate 361
Serotype 23A	Emory isolate 969
Serotype 23F	CDC isolate 2012218064
Serotype 24F	Emory isolate 424
Serotype 25A	Emory isolate 780
Serotype 27	Emory isolate 543
Serotype 28F	Emory isolate 454
Serotype 31	Emory isolate 710
Serotype 32F	Emory isolate 523
Serotype 33F	Emory isolate 531
Serotype 35A	Emory isolate 701
Serotype 35B	Emory isolate 546
Serotype 35B	Emory isolate 385
Serotype 35B	Emory isolate 774
Serotype 35F	Emory isolate 644
Serotype 37	Emory isolate 672

Table 3. TIGR4 DNA Standards and Conversions

Standard	Concentration of Prepared Standard Solution	DNA (wt) / PCR Reaction	Copies / PCR Reaction	Log_{10} Copies / PCR Reaction	Correlation to Bacterial Load in Original Clinical Sample (CFU/mL)
1	400 ng/mL	1 ng	429,000	5.63	85,800,000
2	40 ng/mL	100 pg	42,900	4.63	8,580,000
3	4 ng/mL	10 pg	4,290	3.63	858,000
4	400 pg/mL	1 pg	429	2.63	85,800
5	40 pg/mL	100 fg	42.9	1.63	8,580
6	20 pg/mL	50 fg	21.4	1.33	4,280
7	2 pg/mL	5 fg	2.14	0.33	428

Table 4 *lytA* qPCR Sensitivity Results

***S. pneumoniae* Strain**	**Copies/rxn**	**Log_{10} Copies/rxn**	**No. Replicates**	**No. Positive**	**Detection Rate**
ATCC BAA-334 (TIGR4)	1.07	0.03	20	11	55.0%
	2.14	0.33	20	19	95.0%
	4.28	0.63	20	19	95.0%
ATCC BAA-255	1.07	0.03	20	13	65.0%
	2.14	0.33	20	16	80.0%
	4.28	0.63	20	20	100.0%
ATCC 700676	1.07	0.03	20	19	95.0%
	2.14	0.33	20	18	90.0%
	4.28	0.63	20	20	100.0%
ATCC 49619	1.07	0.03	20	15	75.0%
	2.14	0.33	20	19	95.0%
	4.28	0.63	20	20	100.0%
ATCC 33400	1.07	0.03	20	17	85.0%
	2.14	0.33	20	18	90.0%
	4.28	0.63	20	20	100%

Note: Highlighted rows indicate the LOD (≥ 95% detection) for each individual strain.

Table 5. Precision Testing in the *lytA* qPCR Assay by *S. pneumoniae* Strain

***S. pneumoniae* Strain**	**Copies / Reaction**	**Log_{10} Copies / Reaction**	**Cq Mean**	**Standard Deviation**	**95% CI**
ATCC BAA-334 (TIGR4)	4.0E+05	5.60	19.86	0.307	± 0.14
	500	2.70	29.98	0.333	± 0.14
	5[a]	0.70	36.99	1.012	± 0.45
	4[a]	0.60	37.35	0.891	± 0.39
ATCC BAA-255	4.0E+05	5.60	20.02	0.299	± 0.13
	500	2.70	30.07	0.295	± 0.13
	5[a]	0.70	36.99	0.867	± 0.39
	4[a]	0.60	37.64	0.957	± 0.43
ATCC 700676	4.0E+05	5.60	19.77	0.535	± 0.24
	500	2.70	29.85	0.509	± 0.22
	5[a]	0.70	36.81	0.943	± 0.42
	4[a]	0.60	37.44	1.239	± 0.55
TCC 49619	4.0E+05	5.60	19.88	0.358	± 0.15
	500	2.70	29.88	0.362	± 0.16
	5[a]	0.70	36.78	1.012	± 0.45
	4[a]	0.60	37.24	1.082	± 0.48
ATCC 33400	4.0E+05	5.60	20.09	0.287	± 0.13
	500	2.70	30.21	0.296	± 0.13
	5[a]	0.70	37.38	1.102	± 0.49
	4[a]	0.60	37.72	1.033	± 0.46

a. Undetected replicates at these concentrations were converted to the maximum cycle number (40) for analysis.

Table 6. Summary of Precision Testing in the *lytA* qPCR Assay

Copies / reaction	**Log_{10} Copies / reaction**	**Detection Rate**	**Cq Mean**	**Standard Deviation**	**95% CI**
4.0E+05	5.60	100.0%	19.92	0.385	± 0.07
500	2.70	100.0%	30.00	0.389	± 0.08
5[a]	0.70	96.7%	36.99	1.008	± 0.20
4[a]	0.60	95.1%	37.48	1.057	± 0.21

a. Undetected replicates at these concentrations were converted to the maximum cycle number (40) for analysis.

Table 7. Accuracy Panel *lytA* qPCR results

Type	Source	*lytA* qPCR Cq (triplicate mean)
Serotype 1	CDC isolate 2012002345	25.66
Serotype 3	CDC isolate 2011212843	26.45
Serotype 4	CDC isolate 2011215139	25.45
Serotype 5	CDC isolate 2010220227	28.03
Serotype 6A	CDC isolate 2012002049	26.33
Serotype 6B	CDC isolate 2012203875	25.98
Serotype 6C	Emory isolate 20	26.32
Serotype 7C	Emory isolate 34	27.39
Serotype 7F	CDC isolate 2012214337	25.57
Serotype 8	Emory isolate 36	25.20
Serotype 9N	Emory isolate 86	24.88
Serotype 9V	Emory isolate 153	23.36
Serotype 9V	Emory isolate 381	25.86
Serotype 11A	Emory isolate 183	26.51
Serotype 11B	Emory isolate 229	26.44
Serotype 13	Emory isolate 711	25.09
Serotype 13	Emory isolate 716	27.19
Serotype 15C	Emory isolate 232	27.60
Serotype 16F	Emory isolate 257	26.35
Serotype 18C	Emory isolate 678	24.99
Serotype 18C	Emory isolate 218	22.60
Serotype 19A	CDC isolate 2012215778	25.25
Serotype 19F	CDC isolate 2012214924	26.22
Serotype 20	Emory isolate 336	28.57
Serotype 22F	Emory isolate 361	25.26
Serotype 23A	Emory isolate 969	25.52
Serotype 23F	CDC isolate 2012218064	26.77
Serotype 24F	Emory isolate 424	24.99
Serotype 25A	Emory isolate 780	26.94
Serotype 27	Emory isolate 543	25.61
Serotype 28F	Emory isolate 454	26.17
Serotype 31	Emory isolate 710	29.81
Serotype 32F	Emory isolate 523	24.79

Type	Source	*lytA* qPCR Cq (triplicate mean)
Serotype 33F	Emory isolate 531	27.37
Serotype 35A	Emory isolate 701	26.58
Serotype 35B	Emory isolate 546	25.65
Serotype 35B	Emory isolate 385	25.35
Serotype 35B	Emory isolate 774	26.38
Serotype 35F	Emory isolate 644	27.20
Serotype 37	Emory isolate 672	30.13