

Use and Clinical Interpretation of Pneumococcal Antibody Measurements in the Evaluation of Humoral Immune Function

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Pneumococcal vaccination is a commonly used technique for assessing the humoral immune status of a patient suspected of having immunodeficiency. Interpretation of what constitutes an adequate response, however, can be challenging. This is due to the complexity of the data generated from serotype-specific assays, historical variations in the assays used to measure pneumococcal antibodies, and varying recommendations on the relevant cut points that define response. In this review, we summarize the historical evolution of assays used for this purpose and discuss the analytical considerations that have influenced published data. We also examine current clinical recommendations for defining an adequate response to vaccination, with a particular focus on the interpretation of serotype-specific data generated by multiplex assays.

Streptococcus pneumoniae is a Gram-positive bacillus which causes a range of diseases, including community-acquired pneumonia, otitis media, sinusitis, and meningitis. Pulmonary manifestations are the most common form of invasive pneumococcal disease, and this organism is responsible for roughly one-third of community-acquired pneumonia cases in the United States (1). Children, the elderly, and immunosuppressed patients are among the groups most susceptible to pneumococcal infection. More than 90 different serotypes of this organism have been identified which differ in the makeup of capsular polysaccharides expressed on their surface. Although the prevalences of different serotypes differ between populations, the majority of clinical disease is caused by a relatively small subset of serotypes (2, 3), a fact which has resulted in the clinical effectiveness of pneumococcal vaccination.

The development of vaccines against pneumococcal capsular antigens has played a major role in reducing the morbidity and mortality associated with pneumococcal infection. A variety of different pneumococcal vaccines have been developed which differ in two primary characteristics: the number of serotypes represented in the vaccine and the antigenic nature of the pneumococcal materials used. While the earliest vaccines were based on a mixture of purified capsular polysaccharides from 14 commonly encountered serotypes, coverage was expanded in later versions to produce the PPSV23 vaccine (Pneumovax; Merck & Company Inc., Whitehouse Station, NJ) that is currently in widespread use. The use of conjugated pneumococcal vaccines is a more recent development. The first such vaccine was licensed in the United States in 2000 (PCV7 [Prenar], Wyeth Pharmaceuticals, Madison, NJ) and contained a mixture of antigens from serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F. PCV7 was the primary vaccine used in pediatric patients in the United States until 2010, when it was largely supplanted by PCV13 (Prenar 13; Wyeth Pharmaceuticals, Madison, NJ), which contained 6 additional serotypes (1, 3, 5, 6A, 7F, and 19A). Because of this spectrum of different available vaccines, the profile of antipneumococcal antibodies (PnAb) found in an individual varies depending on the age of the patient and, in the case of children, on the year in which the child was originally vaccinated.

In addition to their use for infection prevention, pneumococ-

cal vaccines are commonly employed as a tool to functionally evaluate a patient's humoral immune response. The basic approach (described in more detail below) is to measure the level of anti-pneumococcal antibodies (PnAb) in the patient's serum before and after vaccination in order to determine whether an appropriate response has occurred. The evolution of analytical methods used for this purpose has paralleled the introduction of new vaccines, and the analytical characteristics of the various assays have had a direct effect on the currently recommended rules for interpreting clinical results. Although many methods have been used over the years for the measurement of serotype-specific PnAb levels, for the sake of discussion they can be broadly broken down into two categories: individual immunoassays that measure PnAb directed against a single serotype and multiplex assays that measure PnAb levels of several serotypes simultaneously.

IMMUNOASSAYS AGAINST INDIVIDUAL PnAb SEROTYPES

The majority of early studies measuring serotype-specific PnAb levels utilized immunoassay techniques. One of the most influential early tests was a radioimmunoassay (RIA) developed by Schiffman et al. (4). In that assay, immunoprecipitation of patient serum was performed using ¹⁴C-labeled pneumococcal polysaccharides and PnAb levels were determined based on the amount of radioactivity present in the precipitant. This assay (or modifications of it) was widely used in early publications, with the later development of nonradioactive enzyme-linked immunosorbent assay (ELISA) techniques occurring along the same lines (5). A lack of readily available standards, however, limited the widespread application of results from such studies

Accepted manuscript posted online 17 December 2014

Citation Daly TM, Hill HR. 2015. Use and clinical interpretation of pneumococcal antibody measurements in the evaluation of humoral immune function. *Clin Vaccine Immunol* 22:148–152. doi:10.1128/CVI.00735-14.

Editor: C. J. Papsian

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and made it difficult to compare results generated by different assays. In addition, the majority of these early assays reported results in units of “ng antibody nitrogen/ml” and established their clinically relevant cut points accordingly (6, 7). This led to later difficulties in attempting to bridge historical results to the more modern assays.

In an attempt to improve the correlation of PnAb results across different laboratories, the World Health Organization published protocols describing standard methods for developing and validating serotype-specific PnAb assays (<http://www.vaccine.uab.edu>) and produced a set of reference standards and calibration materials for use in PnAb assay development (8). PnAb levels in this reference human serum pool (89-S) were assigned in weight-based units ($\mu\text{g PnAb/ml}$), which improved the ability to compare results between assays and between serotypes within an assay. The 89-S reference material rapidly became the standard in the field and has been widely used to demonstrate analytical accuracy for newly developed tests, although a transition to a new lot of reference material (007sp) is under way (9).

The analytical shift from ng antibody nitrogen-based results to weight-based units has played a role in the difficulties in establishing defined cutoffs with modern assays, particularly in adult populations. Many of the original studies which established “protective” cutoffs for antibody levels (defined using infection rates in epidemiologic studies) or “adequate response” cutoffs for vaccine studies were performed using assays which reported in antibody nitrogen units. Little published data are available to establish the conversion factor between the two methods of measurement. The most commonly cited example for a conversion factor comes from recommendations published in 2007 which utilized data derived from a personal communication (10). This factor propagated through later publications and is the source of the commonly utilized cut points of 1.0 $\mu\text{g/ml}$ and 1.3 $\mu\text{g/ml}$ described in the clinical section which follows.

MULTIPLEX ASSAYS

Initial clinical application of serotype-specific PnAb measurement was largely limited to academic studies and vaccine development trials, in part because of the effort and expense required to run the multiple individual ELISAs needed to generate a comprehensive serologic response profile. This changed in the early 2000s with the commercial emergence of multiplex immunoassay platforms in clinical laboratories. One of the earliest described multiplex assays for PnAb measurement was developed using a bead-based flow cytometric methodology (Luminex) that measured quantitative levels of PnAb against 14 serotypes from a single assay (11). This test was subsequently validated using clinical specimens (12) and was eventually approved by the FDA and marketed as an *in vitro* diagnostic (IVD) assay in the United States for a period of time. Multiplex pneumococcal assays, including electrochemiluminescent assays and chemiluminescent microarrays, were also developed on other platforms (13, 14). The increased availability of this testing at multiple reference laboratories and the associated reduction in costs and sample requirements greatly expanded clinical utilization of multiplex serologic PnAb measurements in routine practice.

This widespread application of multiplex PnAb assays, however, brought several additional challenges with interpretation. Quantitative comparisons of PnAb levels measured by multiplex

assays were not always consistent with results from WHO-based ELISA methods (15), and the performance characteristics of these assays often did not meet the criteria set in the original WHO document developed for ELISA testing. Intralaboratory comparisons of assays from different laboratories showed substantial variation in quantitative results even when the same basic technology was being utilized (16), although clinical classifications of patients were relatively unaffected by these quantitative variations using published algorithms (17, 18). As a result, interpretation of multiserotype test results could be a challenge for clinicians trying to apply pneumococcal serotype analysis to their practice in different clinical scenarios.

DETERMINING THE INFECTING SEROTYPE IN AN ACUTE OR PAST INFECTION

Although the pneumococcal antibody tests may be used in attempts to identify the serotype involved in an ongoing infection or in a prior infection, as with other serologic assays for bacterial, viral, and fungal infections, in our opinion, this is not the optimal utilization of the test (19). Testing by isolating the offending pathogen and analyzing the strain involved by serotyping or molecular typing is always optimal. If this is not done during the active infection, then a broad-based pneumococcal antibody test with at least 14 to 23 serotypes may be employed in an attempt to determine the infecting serotype. In this case, a baseline serum specimen should be drawn as soon as possible and should be tested along with follow-up samples collected approximately 2 weeks and 1 month after the initial infection. If a very high antibody concentration corresponding to one serotype is detected or, ideally, if 2-fold to 4-fold increases in concentrations between an early specimen and a specimen drawn 2 to 4 weeks later can be detected, then one can feel fairly confident this result represents a serologic response to the infecting strain. This, of course, assumes that the samples are absorbed with either C polysaccharide and pneumococcal-serotype 22 to remove non-type-specific, cross-reacting antibodies (11, 20) or CWPS-multi (Statens Serum Institute, Copenhagen, Denmark), a product which retains the ability to measure PnAb directed against serotype 22. Again, this is not a strongly recommended use of the assays, except perhaps in cases of outbreaks of pneumococcal disease where serologic studies of those infected might help in identifying the offending serotype. However, isolation and typing of the organism are more reliable.

UTILIZATION OF THE PNEUMOCOCCAL TESTS IN ATTEMPTS TO DETERMINE A PROTECTIVE ANTIBODY CONCENTRATION

Another important application of these assays has come from large clinical studies of vaccine efficacy employing either the 23-valent polysaccharide vaccines or the 7-valent and 13-valent conjugated vaccines (21–23). In these studies, prevaccination and near-term postvaccination samples (collected at time points ranging from 1 to 12 months after vaccination) are generally tested, along with residual antibody concentrations collected at time points ranging from 1 to 5 years after vaccination. The number of individuals in a vaccinated group who develop sepsis, meningitis, or pneumonia, as well as milder infections such as sinusitis and otitis, is determined and compared to the numbers of matched, unvaccinated controls or controls vaccinated with other control vaccines to determine clinical efficacy. In addition, serum samples should be collected from subjects vaccinated with the pneumococcal vaccine who acquired infection as well as from those who

did not. Determinations of serotype-specific antibody concentrations are then utilized in attempts to determine a “protective concentration” of specific antibody.

In general, most of the researchers who conducted these studies have agreed that a pneumococcal-serotype-specific concentration of between 1 and 1.5 $\mu\text{g/ml}$ at 1 month postvaccination results in long-term protection in infants (<2 years old), older children, and adults. In addition, antibody studies of infected versus noninfected vaccinated infants at periods have indicated that the concentrations protective against such pneumococcal infections in infants receiving the PCV7 conjugated vaccine in the United States have been as low as $\geq 0.15 \mu\text{g/ml}$ in studies of the 7-valent conjugated vaccine (21–24). Interestingly, studies carried out in the United Kingdom with the vaccine indicated that higher concentrations of $\geq 0.35 \mu\text{g/ml}$ to $\geq 0.5 \mu\text{g/ml}$ are required for protection (25, 26). The actual scientific evidence for these protective concentrations, however, may not have been developed on the basis of purely sound scientific data. Paris and Sorenson et al. arbitrarily defined a postimmunization antibody concentration of $\geq 1.3 \mu\text{g/ml}$ to be protective in 1998, but this was primarily based on personal communications rather than large data sets (10, 27). Given the variability of the various assays, including the radioimmunoassays, ELISA-type assays, and multianalyte assays currently in use by most of the major reference laboratories, it is reasonable to assume that long-term protection probably does result from a 1-month postvaccine concentration of between 1 and 1.5 $\mu\text{g/ml}$. Arguably, the minor differences between 1.0, 1.3, and 1.5 seem irrelevant given the inherent variability of the most commonly utilized assays, especially for comparisons of results from different reference laboratories (18). Moreover, true responders usually have much higher responses to most serotypes than the minimal 1.0 to 1.5 $\mu\text{g/ml}$, in our experience.

UTILIZATION OF PnAb TESTING TO DETERMINE THE ABILITY OF A POSSIBLY IMMUNE-DEFICIENT PATIENT TO MAKE ANTIBODY

In our opinion, the most important reason for ordering pneumococcal antibody testing is to assess the ability to generate a specific polysaccharide antibody response in patients who have clinical findings suggestive of immune deficiency (i.e., unexplained or recurrent infections and low immunoglobulin levels). A special-interest section of the American Academy of Allergy, Asthma, and Immunology (AAAAI) published in 2012 a working group report which points out many of the inconsistencies in interpretations of pneumococcal antibody testing and recommends approaches to the routine use of these assays (28). It is clearly pointed out that in most cases, patients over 2 years of age who are suspected of having an immune deficiency because of serious sinopulmonary, bloodstream, or central nervous system infections or lowered levels of immunoglobulins or IgG subclasses should be immunized with the 23-valent pure-polysaccharide vaccine, with samples collected pre- and postvaccination (1 to 6 months) for testing. The PCV7-conjugated 13 valent vaccine is, of course, now recommended for all children less than 2 years of age, who often have low IgG2 concentrations and fail to respond to pure-polysaccharide vaccines. In addition, the conjugated vaccine can be used to prime an older nonresponder with respect to the pure-polysaccharide vaccine (29).

Initial work-up of a possible immune-deficient patient often includes vaccination with protein vaccines, including diphtheria,

tetanus, and influenza vaccines, as well as with one of the pure pneumococcal polysaccharide vaccines and, in some cases, the meningococcal polysaccharide vaccine if available. Ideally, a pre-vaccine sample (taken on the day of vaccination most often) and a 1-month-postvaccine serum sample are tested simultaneously. This gives the clinician valuable information about the antibody responses to each of these antigenic vaccine types. There is significant controversy, however, about what constitutes an adequate serotype-specific antibody response following immunization with the pneumococcal vaccine (10, 21–27). In broad terms, response is usually judged by determining the percentage of serotypes which either show a predefined (either 2-fold or 4-fold) change relative to the corresponding baseline sample or achieve an absolute “protective” concentration of antibody. In general, for the pure-polysaccharide pneumococcal vaccines, a cutoff value of 1 to 1.3 to 1.5 $\mu\text{g/ml}$ in a 1-month-postvaccine sample has been utilized as indicative of protection in various studies. Moreover, if the patient responds to fewer than 50% to 70% of the serotypes examined in the assay, the patient can be classified as being deficient in production of antibodies to polysaccharide-coated bacteria (which cause a majority of our pyogenic bacterial infections). This broad range of possible responses can produce ambiguous characterization of responders versus nonresponders, which complicates the use of this test for determining B cell function. In general, children less than 16 years of age respond to at least 50% of the vaccine serotypes when the pure-polysaccharide vaccine is used, while adults have been said to respond to 70% of the serotypes (28).

In our experience, seeing both children and adults with possible immunodeficiency, there is at least some area for leeway in using these cutoff values and especially for meeting the criteria for 4-fold increases in antibody concentrations in deciding whether a patient truly is antibody deficient and requires treatment with intravenous or subcutaneous immunoglobulin. To some extent, this depends on the level of response to the various serotypes. When concentrations of 5 to 10 or $\geq 20 \mu\text{g/ml}$ are generated in response to some serotypes, it is hard to make a case that the patient is incapable of responding to polysaccharide vaccines. It is our opinion that the practice of medicine, especially in the interpretation of immunologic tests such as the pneumococcal post-vaccine immunologic assays, is an art and that flexibility with respect to cutoff figures and fold increases must be maintained. The nature and severity of the infections in the patients and the maximum responses to certain serotypes as well as the overall percentage of serotypes which a patient adequately responds to must always be taken into consideration. It is our opinion that a concentration of between 1 and 1.5 $\mu\text{g/ml}$ at 1 month postimmunization in all likelihood signifies adequate responsiveness for protection over the long term (approximately 5 years). Responses to between 50% and 70% of the serotypes should also be seen as an indication of a normal immune response, in most cases. Recently, researchers who conducted a collaborative study carried out by three major reference laboratories utilizing two in-house-developed assays and one commercial multianalyte assay reported that there was 79% agreement when a threshold-based ($> 1.3 \mu\text{g/ml}$) algorithm was utilized instead of a 4-fold (57%) or 2-fold (96%) algorithm (30). However, the high concordance between laboratories seen with a 2-fold (96%) increase, which may in some cases actually be a more appropriate cutoff value, must also be noted. Remember that “medicine is an art”!

There is another subset of patients who have perfectly normal IgG, IgA, IgM, and IgG subclass levels who suffer significant polysaccharide-encapsulated bacterial infections, including sinusitis, otitis, pneumonia, and even meningitis, and who fail to respond when immunized with the 23-valent pure-polysaccharide pneumococcal vaccine (28). We have seen a number of these patients and have found that many show no responsiveness at all to the majority of the pneumococcal serotypes or, in some cases, even to the *Neisseria meningitidis* pure-polysaccharide vaccine. This syndrome is designated “specific polysaccharide antibody deficiency” and clearly is a real clinical entity (28). Perhaps some of the patients go on to develop common variable immune deficiency and/or IgA and/or IgG subclass deficiency, but in most this is not apparent. These individuals often require immunoglobulin therapy or antimicrobial prophylaxis, while some respond to vaccination with conjugated pneumococcal vaccine to prime the immune system, followed by readministration of the 23-valent polysaccharide vaccine, which often leads to a reasonable response to an adequate number of the serotypes and protection from infection, in our experience and that of others (29). Most of the symptomatic patients with this form of specific polysaccharide antibody deficiency have failed to respond to any of the vaccine serotypes in the 14-valent or 23-valent pneumococcal antibody assay, in our experience. It should be pointed out, however, that others have suggested that there are patients who respond to some but not other serotypes in the pure-polysaccharide pneumococcal vaccine and have a true antibody deficiency requiring therapy as described above. We have not seen such patients and so cannot comment on serotype-specific polysaccharide antibody deficiency. As one considers immunity to infectious diseases in general, however, it certainly seems within reason that all serious invasive infections, as well as even minor ones, may be the result of an individual, serospecific defect in that individual’s immunity!

In summary, pneumococcal vaccination is a valuable tool in the work-up of potential humoral immunodeficiency cases, and the availability of multiplexed PnAb assays allows this response to be profiled with a great deal of granularity. However, historical differences in the analytical methods used to define thresholds, coupled with interlaboratory variability in current methodologies, can make it challenging to set precise criteria for defining a response. In general, although current assays provide a wealth of quantitative data about individual serotype levels, a qualitative assessment of the overall PnAb pattern is arguably the most important factor in determining the adequacy of a response. Because patients with true humoral immunodeficiency usually show a widespread failure of response across virtually all serotypes, small differences in a subset of PnAb tend to have little clinical meaning in a patient who shows a response to multiple other serotypes. While the use of published algorithms can help with these assessments, it is important not to become too fixated on specific quantitative values for any individual serotype at the risk of losing sight of the forest for the trees.

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Harry R. Hill is Professor of Pathology, Pediatrics and Internal Medicine. He serves as the Head of Clinical Immunology/Immunodeficiencies at the University of Utah School of Medicine. After obtaining his M.D. degree from Baylor College of Medicine, he received clinical residency and immunology fellow training at the University of Washington in Seattle. Dr. Hill then trained in host resistance, infectious diseases, and clinical laboratory medicine at the University of Minnesota. He was also an Epidemic Intelligence Service officer with the Communicable Disease Center assigned to the Streptococcal Disease Laboratory in Fort Collins, Colorado, for 2 years. He has been funded by the NIH for a total of 40 years as a principal investigator (PI) or co-PI for work on group B and group A streptococcal disease and rheumatic fever and for various aspects of the role of neutrophils in disease processes as well as in the molecular diagnosis of primary immunodeficiency diseases. He has 265 peer-reviewed articles and 159 text book chapters and review articles (total, 424). His clinical work involves seeing mostly adult and some pediatric patients with primary immunodeficiencies and being the Medical Director of the Cellular and Innate Immunology Laboratory at ARUP Laboratories, the national esoteric reference laboratory owned by the University of Utah. On a lighter note, to keep on moving, Dr. Hill has run 21 marathons, ridden a number of 100-mile bike races and 9 to 10 Snowbird Hill Climbs (3,000 feet of ascent in 10 miles) and more recently (in his 60s and 70s) has climbed most of the major peaks in the Teton Mountains of Wyoming. He also enjoys backcountry skiing. His research laboratory is investigating the role of cytokines, the hormones of the immune system, in inflammation and disease, including coronary artery disease and autoimmune disorders such as rheumatoid arthritis and multiple sclerosis, and, more recently, Dr. Hill has been investigating, along with his colleagues, the molecular causes of primary immune deficiencies using next-generation sequencing.

