

Enzyme-Linked Immunosorbent Assay for Quantitation of Human Antibodies to Pneumococcal Polysaccharides

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Streptococcus pneumoniae is a major human pathogen causing pneumonia, sepsis, meningitis, and otitis media (12). It causes infections most often in young children (12) and elderly adults (1) because their immune systems are either unprepared or unable to respond effectively to pneumococci. In addition, patients with certain chronic conditions like cardiovascular diseases, pulmonary diseases, diabetes mellitus, various cancers, and sickle cell anemia (55) and immunosuppressive conditions, including human immunodeficiency virus infection (23, 47), and cigarette smokers (40) have a greater risk of severe pneumococcal disease.

The major virulence factor of *S. pneumoniae* is the polysaccharide capsule, which reduces phagocytosis by host phagocytes (4). So far, 90 capsular polysaccharides (PS) have been identified by their induction of serotype-specific antibodies (17). Serotype-specific anti-capsular PS antibodies have been shown to provide serotype-specific protection. Passive transfer of the antibody into recipient mice protects the mice from lethal challenge with virulent pneumococci (26, 28). Serotype-specific immune sera were used to treat patients with pneumococcal infections in the preantibiotic era (8).

Because the antibodies to capsular PS are highly protective, efforts to develop pneumococcal vaccines have focused on the use of various combinations of the most commonly identified pneumococcal capsular PS as immunogens. In 1977, a 14-valent vaccine, Pneumovax (Merck, Sharp and Dohme), was licensed for use in older adults and high-risk children >2 years of age. The vaccine contained 50 µg of each of the 14 PS serotypes that represented 80% of the isolates of *S. pneumoniae* from patients with pneumococcal bacteremia at 10 major hospitals in the United States (19). This vaccine was superseded in 1983 by the 23-valent vaccines Pneumovax 23 (Merck, Sharp and Dohme), PnuImune (Wyeth), and Pneumo23 (Aventis Pasteur MSD). These vaccines contain 25 µg of each of 12 of the original 14 PS serotypes plus an additional 11 serotypes.

Because these PS vaccines were not effective in young children (49), a seven-valent pneumococcal conjugate vaccine was developed (30, 59). This vaccine contains the capsular PSs

from the seven most prevalent serotypes causing invasive pneumococcal disease in young children (serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F), each conjugated to a protein carrier, the nontoxic diphtheria toxin mutant CRM₁₉₇. The seven serotypes cause more than 80% of the pneumococcal disease observed in young children in the United States (72). Following the successful demonstration of its efficacy against invasive pneumococcal disease in young children (5, 48), the conjugate vaccine was licensed as Prevnar (Wyeth) and introduced for clinical use in the United States in 2000.

However, the conjugate vaccine requires multiple injections in infants, its effectiveness in the elderly has not been established (44, 58), and its utility in this and other age groups is under evaluation. Although there are efforts to develop pneumococcal protein antigens as vaccines (6, 37), the conjugate vaccines will be improved by adding additional serotypes in order to improve disease coverage outside of the United States (15, 16, 34, 45; J. Nurkka, M. Malm, A. Holm, J. Poolman, C. Laferriere, P. Peeters, H. Käyhty, and T. Kilpi, Abstr. 3rd Int. Symp. Pneumococci Pneumococcal Dis., abstr. P-07, 29a, 2002). Additional changes may include the use of novel adjuvants to enhance the immune response (7, 21, 22, 66), in particular in newborns (20), and combination of the pneumococcal vaccine with other vaccines to minimize the number of injections given in childhood. These new or modified pneumococcal vaccines would likely be evaluated by assessment of their immunogenicity. Thus, there have been extensive efforts to develop assays for quantitation of pneumococcal antibodies. Here, we review the history of the pneumococcal antibody enzyme-linked immunosorbent assay (ELISA) and describe a reliable ELISA procedure that was used in the evaluation of the approved seven-valent pneumococcal conjugate vaccine.

HISTORY

The original assays used to quantify the level of circulating antibodies to pneumococcal capsular PS were based on the Farr assay, a radioimmunoassay that measures antibody binding to radiolabeled capsular PS (56). However, the Farr assay is impractical to support assessment of thousands of specimens associated with clinical trials. It consumes large volumes of sera for each serotype, uses radioactive isotopes, and is not informative relative to the isotope being elicited by the vaccine. Furthermore, it was not clear whether the Farr assay provided

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the necessary serotype specificity (36, 39, 67). Thus, in the early 1980s, the ELISA became the preferred method for estimating antibody concentrations.

In studies using ELISAs, results showed a poor correlation of antibody concentration with the efficacy of the vaccines and animal passive protection. These first-generation ELISAs were later found to overestimate the true anti-capsular PS antibody concentration. The primary reason was that the assay measured antibodies to pneumococcal cell wall PS (C-PS), as well as anti-capsular PS antibodies (29). This occurred because "purified" capsular PS contains up to 5% (by weight) C-PS, which may be covalently bound to the serotype-specific PS via a peptidoglycan moiety (61). Also, most people have antibodies to C-PS, perhaps in response to pneumococcal carriage or infection (13, 29).

Once the problems with antibodies to C-PS were recognized, a second-generation pneumococcal ELISA was developed by taking steps to neutralize C-PS antibodies in test serum samples prior to ELISA measurements. Two different approaches were developed to reduce the impact of nonspecific antibody binding in the ELISA. These approaches were preadsorption either with highly purified C-PS (available from Statens Serum Institut, Copenhagen, Denmark) or preadsorption with a cruder cell wall preparation from a nonencapsulated serotype (29, 35, 60). Wyeth Laboratories used the crude preparation for their vaccine evaluations (46). This simple alteration resulted in better quantitation of the serotype-specific pneumococcal PS antibodies and also improved the correlation of the serum antibody concentration with immune protection, as measured by opsonophagocytosis *in vitro* (68) and as protection against pneumococcal infections in a murine model (54).

However, the second-generation ELISA was found to have insufficient specificity when serum samples from unimmunized adults were investigated (11). Following the discovery that ELISA specificity could be further improved when the test sera were preadsorbed with an irrelevant pneumococcal capsular PS (71), a third-generation ELISA was devised. For the third-generation assay, test serum samples are preadsorbed with C-PS, as well as pneumococcal type 22F capsular PS (10). Serotype 22F was chosen for this purpose because the capsular PS is readily available and is not likely to be included in any future conjugate vaccines.

This third-generation assay format was adopted by experts at a meeting held in 2000 at the World Health Organization (WHO) headquarters in Geneva, Switzerland. Although different assay protocols may be acceptable, the group at Geneva decided that it would be useful to select one well-characterized pneumococcal ELISA protocol as a reference. Since the performance-based approach is chosen, the selection of one specific protocol is not meant to limit choices but to provide guidance for new laboratories developing ELISAs to evaluate responses to pneumococcal vaccines. At that meeting, participants chose 12 calibration serum samples with known, assigned antibody concentrations to be used in pilot experiments by laboratories wishing to perform pneumococcal antibody ELISAs. The participants also defined a criterion for acceptance of the results of the calibration serum sample analysis: the results of a new ELISA should exhibit a percent error of 40% or less compared to the assigned values for 9 of the 12

calibration serum samples. This criterion would be applied to assay results for each serotype (41).

Two reference laboratories were established with funding from the WHO to help other laboratories set up or troubleshoot their pneumococcal ELISA. The WHO reference laboratories are currently located at the Institute of Child Health in London, England, and at the National Institutes of Health (NIH) Pneumococcal Reference Laboratory at The University of Alabama at Birmingham. Additional descriptions of the decisions by these experts are available through the web at www.vaccine.uab.edu. Later, at the Third International Symposium on Pneumococci and Pneumococcal Diseases, held in 2002 in Anchorage, Alaska, the earlier points were reaffirmed and it was also recommended that assays to assess antibody function should be used to supplement ELISA antibody concentration measurements (25).

GUIDANCE PROTOCOL FOR THE THIRD-GENERATION PNEUMOCOCCAL ANTIBODY ELISA

The details of the guidance protocol are important to ensure the success of the analysis. Some of the more important details are given in the next section of this report; however, explicit details for developing and using the pneumococcal antibody ELISA are given in the web document with the title Training Manual for Enzyme-Linked Immunosorbent Assay for the Quantitation of *Streptococcus pneumoniae* Serotype-Specific IgG (Pn PS ELISA) (<http://www.vaccine.uab.edu>). In this section, we describe an overview of the guidance procedure used to quantitate anticapsular immunoglobulin G (IgG) in serum samples, which is an ELISA using pneumococcal capsular PS-coated ELISA plates. It has evolved from the methods described by Quataert et al. (32, 46) and Concepcion and Frasch (9).

Briefly, the guidance procedure for the Pn PS ELISA suggests coating each well of a medium-binding microtiter plate (e.g., Costar 9017 or equivalent) with 100 μ l of the serotype-specific pneumococcal PS antigen (American Type Culture Collection [ATCC], Manassas, Va.) and incubating it at 37°C for 5 h in a humidified chamber. The coated plates are washed by soaking for 30 s with 1 \times Tris-buffered saline–0.01% Brij 35 solution (pH 7.2) and washing five times with the same buffer. The serum reference assay standard (89-SF) is adsorbed with C-PS, but all other samples (quality control [QC] specimens and test specimens) are adsorbed with optimal concentrations of C-PS and 22F. Note that, unlike serum samples and QC samples, the 89-SF standard is only preadsorbed with C-PS (not 22F) because the serotype-specific antibody concentrations for 89-SF were determined without 22F adsorption. After the preadsorption step (30 min), the serum specimens are serially diluted and added to the microtiter plate (50 μ l/well) following a predetermined template. Some wells in the microtiter plates have no serum specimens in order to monitor nonspecific background binding in the assay. Serum specimens are incubated in the PS-coated microtiter plates for 2 h at room temperature. The plates are washed as described above, and 50 μ l of diluted goat anti-human IgG-alkaline phosphatase conjugate is added to each well.

The plates are again incubated for 2 h at room temperature and washed as described above. Finally, the substrate is added

(100 μ l of 1-mg/ml *p*-nitrophenyl phosphate) and the mixture is incubated for 2 h at room temperature. The reaction is stopped by the addition of 50 μ l of 3 M NaOH to all of the wells, and the optical density at 405 nm is measured with a reference filter of 690 nm. Optical density data are converted to antibody concentrations with a computer program like CDC ELISA (described below), which uses a four-parameter logistic-log method to perform a curve-fitting procedure. Explicit details of the guidance procedure are provided at <http://www.vaccine.uab.edu>. In general, the detection limit of the guidance pneumococcal antibody ELISA is about 0.01 mg/liter and the interassay coefficient of variation is about 30%.

KEY REAGENTS FOR THE PNEUMOCOCCAL ANTIBODY ELISA GUIDANCE PROTOCOL

Consistent with the validation of any method, preliminary tests should be completed to select optimal reagents, concentrations, and conditions (32). The performance of the ELISA is critically dependent on several key reagents. The purity of the water used is extremely important, and only type I water (sterile, endotoxin free) should be used. For critical steps, it may be easier to use commercially available bottled pyrogen-free water. The storage of water is important because microorganisms can grow during long-term storage, and their products can cause erroneous results.

Reference serum 89-SF, the antipneumococcal standard reference serum, is a human reference standard derived from the pooled sera of 17 adults immunized with a 23-valent pneumococcal capsular PS vaccine (PNU-IMUNE; Lederle), a meningococcal PS vaccine (MENOMUNE; Connaught), and a haemophilus conjugate vaccine (ProHIBIT; Connaught) (46). 89-SF is available in lyophilized aliquots as U.S. standard reference serum lot 89-SF, from Carl Frasch, Center for Biologics Evaluation and Research, U.S. Food and Drug Administration. The standard antibody concentrations were assigned by ELISA where preadsorption with a cell wall preparation composed primarily of C-PS was used. These standard values for the serotype-specific antipneumococcal antibody concentrations for 89-SF are given at www.vaccine.uab.edu/values89-sf.htm.

A set of serum specimens with known antibody concentrations (referred to as calibration sera) can be obtained from the National Institute of Biological Standards and Control in England by contacting David Goldblatt at the Institute of Child Health, Hertfordshire, England. The calibration sera (41) are composed of 12 serum samples from adults obtained before and after vaccination with a 23-valent pneumococcal PS vaccine (PneumovaxII; Aventis Pasteur MSD). Additional details and the standard antibody concentrations for nine pneumococcal serotypes (1, 4, 5, 6B, 9V, 14, 18C, 19F, and 23F) are given at www.vaccine.uab.edu/refer/qc3.pdf. The calibration sera are only to be used to demonstrate the comparability of an ELISA procedure as a part of assay development.

For routine use, each laboratory will need to prepare a supply of sera for everyday QC determinations. These QC pools can be prepared from outdated blood from the blood bank or from sera collected from volunteers who were immunized with a pneumococcal PS vaccine. Usually, high- and low-titer sera can be found by screening outdated units of blood. If suitable plasma units are identified, they must be

converted to serum for use in the ELISA. Human serum pools are commonly used as QC sera after their antibody levels have been well characterized. To be sure that the ELISA is accurate across a variety of antibody concentrations, some QC sera should have high and some should have low antibody concentrations. There should be one QC serum sample per ELISA plate (some laboratories use more than one) and at least one QC sample with high antibody concentrations representing multiple serotypes for every three assay runs. All QC samples should be assayed enough times to allow the investigator to establish an expected range of concentrations for low-, moderate-, and high-antibody QC samples to ensure that the ELISA consistently produces acceptable results.

An ideal QC (calibration or reference) serum should be obtained from a population identical to the test population. However, the calibration serum samples discussed here were prepared from adults immunized with a PS vaccine. There may be some differences in the behavior of serum specimens from other test populations or those immunized with different vaccines. For example, most studies of children have used conjugate vaccines and, further, children may produce antibodies with antigen binding affinity lower than that of those produced by adults (18, 57), or there may be differences between adults and elderly adult populations (14, 51). Therefore, any analysis must consider these issues and the potential effects on the conclusions of the study.

Purified pneumococcal PSs, manufactured by Merck, are distributed by ATCC, which offers 24 PS types, 23 of which are the pneumococcal PS types that are included in the 23-valent vaccines. The 23 serotypes found in the vaccine are the Danish serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, and 33F. Note that the various pneumococcal PS serotypes are identified by two different systems; a U.S. system and a Danish system, although the Danish system is the currently accepted system of nomenclature. A conversion table showing the correspondence between the two systems is available at www.vaccine.uab.edu/images/serotypes.pdf and at the ATCC web site (www.atcc.org/Products/PurifiedPneumoPoly.cfm/). The pneumococcal capsular PS should be reconstituted by the addition of sterile water to a final concentration of 1 mg/ml, dispensed, and stored at -20 or -70°C . Each laboratory must predetermine the optimal coating concentration for each Pn PS serotype antigen.

Two adsorbents are used to minimize nonspecific signals that can arise because of antibodies directed against bacterial common antigens. One adsorbent is pneumococcal PS 22F, which can be obtained from the ATCC as described above. The other is purified pneumococcal C-PS, which can be obtained from the Statens Serum Institut in 10-mg vials (article 3459; consult <http://www.serum.com/sw379.asp>). This product is highly purified, freeze-dried C-PS prepared from a nonencapsulated strain of *S. pneumoniae* and has one phosphocholine per repeating unit. The vial should be reconstituted with sterile water to a concentration of 1 mg/ml and stored at -20 or -70°C . As with the Pn PS coating concentrations, the optimal concentrations of C-PS and 22F for preadsorption must be predetermined by each laboratory.

Other materials, reagents, and conditions that may vary between laboratories include the antigen binding capacity of mi-

croter plates, the optimal pneumococcal PS coating concentration, the enzyme-labeled secondary antibody, and the optimal concentration of the enzyme-labeled secondary antibody. All of these parameters must be established carefully before embarking on any large-scale ELISA analysis. Detailed protocols for microtiter plate selection and determination of the optimal PS coating concentration for each serotype, optimal secondary antibodies, and antibody dilutions are given at www.vaccine.uab.edu.

Data analysis procedures can be a significant source of error (43). The four-parameter logistic-log function was found to be preferable for the analysis of ELISA data for antibodies to *Neisseria meningitidis* group A PS (43) and was adopted for use in the *S. pneumoniae* antibody studies. A computer program (called CDC ELISA) was written by Brian Plikaytis at the Centers for Disease Control and Prevention, Atlanta, Ga. This program performs the logistic-log fitting and is useful for calculation of pneumococcal antibody concentrations from ELISA data. It can be downloaded free of charge from www.cdc.gov/ncidod/dbmd/bimb/elisa.htm. Other computer programs are acceptable if they are validated against the CDC ELISA program.

LIMITATIONS OF THE PNEUMOCOCCAL ANTIBODY ELISA GUIDANCE PROTOCOL

The assay described here is optimized to measure IgG, not IgA or IgM, antibodies. Although modifications of the protocol would enable measurements of IgA and IgM antibodies, one must examine the performance of the assay in detail. Also, IgG antibodies against pneumococcal capsular PS are mostly IgG2 in adults and are mostly IgG1 in young children who have been immunized with the conjugate vaccine (31, 70). It is thus important to use a secondary antibody that binds all four IgG subclasses (IgG1, IgG2, IgG3, and IgG4) equally well. Monoclonal antibody clone HP-6043 binds all four subclasses (24) and may be used.

Occasional serum specimens have optical density versus dilution curves that are quite different from those obtained with the standard reference serum (89-SF). In these cases, estimation of the antibody concentration for the specimen depends on the sample dilution, as was found in studies of antibody to *Haemophilus influenzae* type b capsular PS in serum from children (2, 42, 69). Therefore, some (M.H.N. and C.E.F.) use the lowest serum dilution that yields an antibody concentration within the linear range of the standard curve. Further work is needed to understand the basis for this phenomenon. One possibility is that these specimens have antibodies with antigen binding affinities different from those present in 89-SF. In this regard, note that the affinity (or avidity) of antibodies may depend on the age of a person and/or on the type of vaccines used. For instance, young children may produce antibodies with low affinity, and the PS conjugate vaccine may elicit antibodies with a higher affinity than those resulting from a PS-only vaccine. Alternatively, the antibody in the specimen may recognize a unique epitope that is not recognized by 89-SF. It has been shown that an epitope may appear (65) or disappear (63), depending on the type of ELISA plate used (33).

The third-generation assay has been extensively evaluated for assay specificity. Its specificity is not absolute and may not

be sufficient when the assay is used to analyze serum samples from minority populations in the United States or from non-U.S. and/or non-European locations. For instance, sera from Native Americans and Africans may exhibit different analytical characteristics. However, the third-generation pneumococcal antibody ELISA has been shown to be sufficiently specific to be useful in evaluating pneumococcal vaccines and can be used as a starting point for analysis of the IgG response of any population.

To interpret the pneumococcal antibody concentrations obtained by ELISA, one needs to know the pneumococcal antibody levels sufficient for immune protection and the antibody levels that might be anticipated after vaccination. Serotype-specific antibody concentration is generally correlated with opsonophagocytic activity in vitro (10, 38, 50). It is not clear how much antibody is sufficient for protection against pneumococcal infections in vivo. A report stated that the antibody concentration protecting 50% of rats from experimental pneumococcal infections is about 0.1 to 3.5 mg/liter (62). Protective antibody levels may vary depending on the pneumococcal serotype (54) and type of infection, as higher concentrations are needed to clear a lung infection than are needed to prevent bacteremia (53). Furthermore, avidity of the pneumococcal antibodies may affect opsonophagocytosis in vitro (3) and protection against experimental pneumococcal infections (53, 64). Rennels et al. (48) and Black et al. (5) reported pneumococcal antibody levels in young children immunized with a course of seven-valent conjugate vaccines. The observed antibody concentrations in immunized subjects provide a basis for future comparisons, as these antibody levels provided protection from sepsis (5); however, these studies do not define the minimum threshold level associated with protection.

The vaccine response among the elderly was reported by Rubins et al. (52), who studied antibody response to a single dose of a 23-valent PS vaccine with the second-generation ELISA. The preimmune antibody levels for each of the 23 PS serotypes were 1.54 to 8.12 mg/liter, and the postimmune levels were 1 to 15.9 mg/liter, suggesting that, in general, vaccination caused an apparent two- to fourfold rise in antibody levels. In another study, elderly patients with chronic obstructive pulmonary disease vaccinated with either 23-valent PS vaccine or a monovalent 6B-conjugate vaccine showed a significant and comparable increase in antibodies and opsonophagocytosis to 6B, which also correlated significantly in both chronic obstructive pulmonary disease groups (27). While these reports contribute to our understanding of immunity related to *S. pneumoniae*, the response to pneumococcal vaccines is dependent on the vaccinee population and the nature of the vaccine. Additional studies with the ELISA described herein and at the website will help to clarify these issues.

SUMMARY

The pneumococcal PS ELISA has a long and complex history. While the procedure has undergone many refinements over the years, we present a current consensus guidance protocol. This protocol is a web-based document for those who want to use the ELISA to test the immune response to pneumococcal PS and PS-protein conjugate vaccines (<http://www.vaccine.uab.edu>). It contains detailed information on the ma-

terials, reagents, conditions, and procedures to aid those interested in implementing the pneumococcal ELISA that yields results that are directly comparable within and between laboratories.

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