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## **Meeting Report**

# **WHO Workshop on Standardization of Pneumococcal Opsonophagocytic Assay**

**Geneva, Switzerland**

**25-26 January, 2007**



## **Summary**

Pneumococcal disease continues to be a major cause of morbidity and mortality among both the elderly and the very young. The prevention of pneumococcal infections is a prerequisite for achieving the rates of reduction in childhood deaths required to meet targets set as part of the Millennium Development Goals. A safe and effective pneumococcal conjugate vaccine is available and others, which are essential for a healthy marketplace, are under advanced development. Licensure of these new products will depend on the evidence of a functional immune response to vaccination. WHO/IVB/QSS convened a meeting in Geneva, 25-26 January 2007, to review progress and formulate a plan for the international standardization of such a functional assay (Opsonophagocytic Assay, OPA) through a global forum including regulators, academia and industry. It was agreed that a stepwise, iterative, performance-based approach could be useful. Consequently, a three-phased strategy was developed. Sub-groups have been assigned to work on different components. It is anticipated that the product of this process, a standardized "reference" OPA method, will be established by WHO and published in the Technical Report Series to provide guidance to the regulators and manufacturers in the vaccine evaluation for licensure worldwide. This will facilitate the development of new pneumococcal vaccines, in particular in evaluating vaccine efficacy, and make the efficacy data comparable between different vaccines.

## **Background**

Global estimates suggest that approximately ten million childhood deaths per year result from pneumonia and that about 40% of childhood deaths can be attributed to pneumococcal disease. The prevention of pneumococcal pneumonia is therefore a prerequisite for achieving the rates of reduction in childhood deaths required to meet targets set as part of the Millennium Development Goals.

The existing seven-valent pneumococcal conjugate vaccine was licensed following protective efficacy trials in California and subsequently efficacy trials have been completed with nine-valent vaccines in South Africa and Europe. It is accepted that efficacy trials will be impractical for the licensure of further serotypes and higher valency formulations. Instead, licensure will depend on the evidence of a functional immune response to vaccination. There is a substantial and compelling body of evidence that the rapid clearance of pneumococci from the blood is due to opsonophagocytosis mediated by type-specific antibody and complement. Thus, the ability of a pneumococcal disease or vaccination to elicit opsonophagocytic antibodies is widely accepted as evidence of a potentially protective immune response.

The further development of pneumococcal conjugate vaccines by both developed and developing country manufacturers alike requires a robust, standardized opsonophagocytic assay (OPA).

The development of standardized assays and serological criteria for the evaluation and licensure of new pneumococcal vaccines has been long pursued by WHO in collaboration with expert laboratories worldwide. International technical specifications for

pneumococcal conjugate vaccines (1) recommend that, in addition to demonstration of IgG antibody concentration, as measured by ELISA, functional antibody titers (opsonophagocytic antibodies) should be evaluated during clinical development programmes, to support regulatory submissions. In a workshop held in Atlanta in June 2005, progress made for the development of OPAs was reviewed and the standardization issues were extensively discussed by representatives from academia, industry, government agencies and public reference laboratories. A follow up meeting held at the 5th International Symposium on Pneumococci and Pneumococcal Disease (ISPPD) in Alice Springs, Australia in April 2006 reiterated the need for accelerating the process of evaluating and standardizing the approach to OPAs so that a standardized assay could be designated as a reference assay for use worldwide to aid in the evaluation of new pneumococcal vaccines.

In response to this need, WHO organized the Workshop on Standardization of Pneumococcal Opsonophagocytic Assay, in Geneva, Switzerland, 25-26 January 2007, to review current progress of OPA in a broad forum of regulators, academia and industries; discuss regulatory and industry perspectives on the development and use of OPA; formulate a plan for the standardization of the pneumococcal OPA and identify the need of developing international reference reagents.

### **Chairman's introduction**

**Elwyn Griffiths**, Health Canada, Canada

The World Health Organization (WHO) has a long-term interest in the evaluation of pneumococcal vaccines. The WHO has supported the development of the pneumococcal antibody ELISA that is being used widely throughout the world for the evaluation and approval of pneumococcal vaccines. Due to the limitations of ELISA, it is essential to have a standardized assay for measuring pneumococcal antibody function. Such an assay can be used to help develop and approve new pneumococcal vaccines, including formulations containing additional serotypes. It can also be used to develop new applications of the vaccine. The OPA measures the protective function of pneumococcal antibodies. The primary objective of the present consultation was to discuss killing-type OPAs, although other OPAs were also considered.

### **Overview of WHO program/activities in facilitating evaluation of pneumococcal vaccines**

**David Wood**, WHO/IVB/QSS

The WHO is mandated by its member states to establish and promote international standards for biological products such as vaccines. Over more than 50 years, the international biological standardization program at the WHO has produced three types of international standards: written standards (e.g. WHO recommendations or guidelines), biological measurement standards (e.g., WHO International Standards/International Reference Preparations), and the evidence base for the WHO standards. The WHO recommendations for the production and control of pneumococcal conjugate vaccines (1) are used by regulatory authorities, manufacturers, and product users (e.g., UN agencies) for harmonizing evaluation of various pneumococcal vaccines. The same three groups

use the WHO biological standards for comparing pneumococcal vaccines. The purpose of the current meeting was to generate the evidence base for the future standard OPA format.

Setting norms and standards including establishment of reference materials and promoting their implementation is one of the six strategic objectives of the biological standards program for the next 6 years (until 2013). The Quality, Safety and Standards (QSS) team, which is led by Dr David Wood, focuses on meeting current international norms and standards on quality and safety in the use of vaccines and other biological products and immunization-related equipment. Specifically, QSS i) sets norms and standards and establishes reference preparation materials; ii) ensures the use of quality vaccines and immunization equipment by their prequalification and by strengthening capacities of national regulatory authorities; and iii) monitors, assesses and responds to immunization safety issues of global concern. The QSS team is a part of the Department of Immunization, Vaccines and Biologicals (IVB), which is directed by Dr Okwo-bele. Under the Director's office, there are two more teams: the Initiative for Vaccine Research (IVR) and Expanded Programme on Immunization Plus (EPI+) teams, which are, respectively, led by Drs. Kieny and Cherian.

**Thomas Cherian, WHO/IVB/EPI+**

An objective of the Millennium Development Goal 4 (MDG4) is to reduce the death rate of children (<5 years old) by 2 fold. Such a reduction will save more than 40 million children by 2015. Pneumonia is a leading killer of children as it is responsible for one-quarter of the 10 million child deaths per year. Nearly 70 % of the 2 million child pneumonia deaths occur in Africa and South Asia. In addition, about 1 out of 10 child deaths worldwide is due to a pneumococcal disease. Thus, pneumococcal infections should be prevented.

Pneumococcal conjugate vaccines (PCVs) are known to be effective against pneumococcal diseases in children. The use of PCVs has reduced pneumonia deaths in The Gambia by 16%, has reduced pneumonia hospitalization by 25%, and provides herd immunity. They cost about \$22 per disability-adjusted life year (DALY) and saved \$691 per death averted. Their cost effectiveness is higher in countries with a high prevalence of pneumococcal diseases. Thus, although PCVs are considered to be expensive, they meet the WHO criteria for being "very cost-effective." The conjugate vaccines should avert about 4 million deaths by 2025; accelerating their introduction would save 3.7 million additional lives by 2025.

The Global Alliance for Vaccines and Immunization (GAVI) Board approved an investment case for introducing PCVs in 2006 and allocated about \$200 million for the vaccines. In 2006, the Strategic Advisory Group of Experts (SAGE) recommended that a 7-valent PCV (PCV-7) should be used in countries with a mortality rate among children (<5 year old) of >50 deaths per 1000 births or with more than 50,000 child deaths per year [published in The Weekly Epidemiological Record (WER), 1/12/07]. The WHO Global Advisory Committee on Vaccine Safety (GACVS) reaffirmed the safety of PCV-7 and other pneumococcal conjugate vaccines, and their potential to reduce rates of

pneumococcal diseases and overall infant mortality at its meeting in November 2006. The PCV-7 is expected to be introduced to some countries in 2008. PCV-10 is scheduled to become available in 2009, and PCV-13 is expected to become available in 2011. In addition, there are three emerging country manufacturers of PCVs.

Future challenges are to ensure vaccine supply and to measure vaccine impact. Advanced Market Commitment (AMC) creates market without a guarantee of purchase. \$1.5 billion is set aside for AMC funding. As a working hypothesis a guaranteed AMC price of \$7/dose has been mentioned but a final price still needs to be finalized based on bilateral negotiations with manufacturers, but the post-AMC market price is expected to be lower.

In October 2006, WHO consulted experts on the serotype compositions of PCVs. Those experts considered the serotype compositions of PCV-10 and PCV-13 to be adequate. The same experts confirmed that there is a role for multivalent vaccines with serotype composition different from that in the currently licensed PCV7. -The 10-valent and 13-valent pneumococcal conjugate vaccines under development are likely to cover most serotypes causing serious disease worldwide. Some variations in serotypes in different countries were determined to be acceptable. The experts also recommended exploring the use of OPA for clinical evaluations.

## **Current development status of OPA**

**David Goldblatt**, Institute of Child Health (ICH), UK

*Hemophilus influenzae* conjugate vaccines were first licensed in 1988. Later, after problems were noted with the hemophilus antibody assays, a standardized hemophilus antibody ELISA was adopted in 1996. To avoid such problems when pneumococcal vaccines were developed, pneumococcal antibody ELISA workshops began early with a meeting in Atlanta in 1994. As a result, a method for comparing ELISA assays was published in 2000 (2). In 2000, Prevnar was licensed and the Wyeth ELISA was accepted as a guidance protocol and WHO created two pneumococcal serology reference laboratories. In 2002, the ELISA protocol was published online ([www.vaccine.uab.edu](http://www.vaccine.uab.edu)) and described in a journal in 2003 (3).

To assist the standardization of pneumococcal antibody ELISA, 24 pairs of quality control (QC) sera were prepared in 1996. Originally, 40 persons were vaccinated with a 23-valent pneumococcal polysaccharide vaccine. Then 300 ml of pre-immune plasma and 600 ml of post-immune plasma were obtained from each person by plasmapheresis. After converting the plasma to serum, pre-immune and post-immune sera from 16 donors were pooled to prepare a secondary pneumococcal standard for Europe. Paired sera from the remaining 24 donors were used as the QC sera. Among 48 sera (i.e., 24 pairs), 12 sera were chosen as the QC panel for the ELISA standardization. Their titers were established in a collaborative study, and 2-ml aliquots were prepared for distribution worldwide.

The standardization of pneumococcal OPA has already begun. In 2003, a multilaboratory evaluation of the CDC protocol was performed and described in a publication (4). In 2005, a meeting was held in Atlanta, with the meeting summary being published (5). In addition, three papers describing a well-characterized OPA were published in 2005-2007 (6-8). Since details of the OPA assay used will likely vary between laboratories, WHO may have to develop performance-based criteria for OPA standardization. The standardization would be multi-stage and would require standard and/or QC sera.

**George Carlone, Center for Disease Control and Prevention (CDC), USA**

Since passive administration of antisera was found to protect rabbits against pneumococcal infections in 1891, serum from immune horses has been used for treating patients with pneumococcal infections. The active components of immune sera are serotype-specific antibodies, which can opsonize pneumococci for phagocytes by fixing complement. The phagocytes can then rapidly clear the opsonized pneumococci. The opsonic antibodies can be induced by natural infections or by vaccination.

Early *in vitro* assays for opsonization visualized bacterial uptake (9) or measured complement consumption (10). Later assays measured the uptake of radiolabeled bacteria (11) or chemiluminescence (12). In the 1990's, the classical killing assay (13) and the flow cytometric uptake assay were developed to evaluate serological responses to pneumococcal vaccines.

The current gold standard of pneumococcal antibody OPA is the viable cell-killing assay described by the CDC in 1997 (13). That assay has several desirable features, such as its micro-scale, its use of standardized target bacteria, and its use of a phagocytic cell line (HL-60), which can be standardized and requires no blood donors. However, it has several shortcomings. It is slow and requires relatively large serum volumes. A multi-laboratory evaluation of this assay established that the gold standard OPA can be a killing assay based on rabbit complement and HL-60 cells (4).

Recently, several multiplex OPA formats have been developed. Two types of multiplexed killing-type OPA were developed using antibiotic-resistant target bacteria. One OPA uses rapid colony counting as the assay readout (7), and the other OPA uses fluorescence as the readout (14). The latter assay measures the fluorescence of alamar blue dye, which reflects the metabolic activity of the surviving living bacteria (described more by Dr Romero-Steiner below). In addition to the killing-type OPA, a multiplex "uptake" OPA has been developed, which uses a flow cytometer to measure the uptake of fluorescent target bacteria or antigen-coated plastic particles (15).

Pneumococcal opsonic activity has been shown to correlate with protection, and the gold standard OPA is the viable cell killing assay. Before OPA becomes routinely used, a multiplexed killing or uptake assay should be further standardized and validated with appropriate serum specimens. To decide on the gold standard OPA, a multi-laboratory study is needed to compare all the available OPAs. An end user should be able to select any OPA format as an operational OPA as long as it generates results correlating to those established by the gold standard.

**Moon Nahm**, University of Alabama at Birmingham (UAB), USA

Since the killing-type OPA from the CDC (13) is the *de facto* gold standard in the field, the assay was further characterized and improved in several aspects to make it more reliable and rapid (7). First, it was found that HL-60 cells from a single source (American Type Culture Collection, ATCC) should be used. Second, bacterial colony counting was automated using an agar overlay containing 2,3,5,-triphenyltetrazolium chloride (TTC) dye. The use of TTC also miniaturized the assay and reduced the production of contaminated biowaste. Third, the assay parameters were optimized to reduce non-specific killing and improve reliability. In addition, the OPA was converted to a multiplexed OPA by using antibiotic-resistant target bacteria (7, 16). The multiplexing has greatly reduced the amount of serum required for the assay and is very easy to implement for any laboratory performing a conventional killing OPA. A 4-fold multiplexed OPA (MOPA4) has been extensively characterized as showing both specificity and sensitivity. Also, MOPA4 can be routinely performed on a large scale, with the assay throughput of MOPA4 being equivalent to that of ELISA.

At present, a method of automated data analysis is being investigated. Instead of providing discrete results (classical “titers”), the new method would give continuously variable results by interpolation. The continuous results should be more precise and accurate. To improve the long-term stability of MOPA4, acceptance criteria are being developed for assay components such as phagocytes, target bacteria, and complement. The acceptance criteria as well as a detailed assay protocol for MOPA4 are available from the following Web site: ([www.vaccine.uab.edu](http://www.vaccine.uab.edu)). In addition, the target bacteria for MOPA4 are readily available and performing MOPA4 requires no special tools. Thus, MOPA4 is ideally suited to be a reference assay.

**Roland Fleck**, National Institute for Biological Standards & Control (NIBSC), UK  
OPA requires a reliable supply of phagocytes. While many cell lines can potentially be used as phagocytes, the NB-4 and HL-60 cell lines have already been used as OPA phagocytes. HL-60, which was derived from a patient with acute promyelocytic leukemia, can be relatively easily maintained in culture and differentiated into phagocytes with DMF, DMSO, or ATRA. In addition, HL-60 cell differentiation can be monitored by surface phenotype changes. Consequently, HL-60 cells have been extensively used for OPA. The use of HL-60 cells as OPA phagocytes has been recently reviewed (17).

HL-60 cells from the ATCC in the U.S.A. work for OPA, but HL-60 cell lines from different cell banks throughout the world may not. Thus, it is highly desirable to have one cell bank providing authenticated and characterized HL-60 cells for use with OPA. That cell bank could also ensure that HL-60 cells are of the same age (passage number), are free of pathogens and contamination, and have an established differentiation potential. The National Institute of Biological Standards and Controls (NIBSC) in England, the WHO International Laboratory for Biological Standards, will seek to create a HL-60 cell bank in collaboration with U.S. FDA.

**Sandra Romero-Steiner**, CDC, USA

Alamar blue (AB) is a dye whose fluorescence is proportional to the number of metabolically active bacteria in a reaction well. AB has been used for serum bactericidal assays of antibodies to hemophilus and meningococci. To develop a multiplexed OPA that requires no colony counting and that could be completed within a day, AB has been recently adapted to a 7-fold multiplexed pneumococcal antibody OPA using antibiotic-resistant target bacteria (14). Also, to achieve a high assay throughput, many assay steps have been automated using robots. However, some assay conditions have been significantly modified from the killing-type CDC OPA. For instance, the E:T ratio (effector to target cell ratio) was only 11 (instead of 400). When its analytical performance was compared with the killing-type CDC OPA using 24 adult sera, its correlation coefficients ranged from 0.76 to 0.97 for seven serotypes. Although this assay can provide a totally automated OPA, other laboratories found that the assay results can vary depending upon the sera being tested. Additional validation of this technique is needed with sera from infants and high risk populations to demonstrate its utility in vaccine evaluations.

**Isabelle Henckaerts**, GlaxoSmithKline Biologicals (GSK), Belgium, Representative from International Federation of Pharmaceutical Manufacturer's Association (IFPMA)

GSK has developed a killing-type OPA for 13 serotypes by modifying the original killing-type CDC OPA. The modifications were made to improve assay productivity, sensitivity, and robustness. For instance, before the robustness of the assay was improved, assay sensitivity could fluctuate up to 10 fold. Improving the robustness was primarily achieved by selecting target bacteria, by adapting bacterial working seed dilution, and by adjusting rabbit complement concentration for each lot. GSK now uses serotype 6A and 6B strains from the CDC, a serotype 3 strain from the Statens Serum Institute in Denmark, and a 19A strain from the National Public Health Institute (KTL) in Finland, with all other bacterial strains being in-house GSK clinical isolates maintained via master-working seed systems. To obtain the results, GSK interpolates the data by using a 4-parameter logistic fit to obtain "continuous titers" instead of "discrete titers" and normalizes the results to its control sera. The GSK OPA has a high throughput (~500 valid OPA results/person/month) and is reproducible (CV<30%), repeatable (CV<25%), and linear (CV<30%) for the 13 serotypes. The variation between the beginning and the end of the assay has a CV of <30%. The GSK OPA has difficulty with serotype 3 with origins other than Statens Serum Institute due to the mucoid nature of its colonies.

The GSK OPA is clinically validated for nine serotypes (seven in Prevnar plus 6A and 19A) using sera from children immunized with Prevnar. GSK found that % OPA  $\geq$  1:8 correlate better with published efficacy data than ELISA. The amount of ELISA antibody concentration providing the "threshold opsonic activity" was determined and varied for different serotypes. For example, serotype 19F requires a high antibody concentration and ELISA overestimates the clinical impact whilst for serotypes 6A, 6B and 23F, GSK found that ELISA underestimates protection.

**Branda Hu**, Wyeth Vaccine Research, USA, Representative from IFPMA

OPA measures the opsonophagocytic function of antibodies and will provide a better surrogate for protection in assessing pneumococcal vaccine in adults. Four biological and labile components (target bacteria, phagocytes, complement, and human antibodies in test sera) are involved in the OPA. To achieve assay consistency in OPA, these components must be tightly controlled. Target bacteria are harvested in late log phase because they give the most consistent and robust assay performance. Bacteria harvested in mid log phase may result in high non-specific killing and those harvested at the stationary phase may yield low assay sensitivity. HL-60 cells should be carefully monitored. Incomplete killing of target bacteria was observed with high passage of differentiated HL-60 cells and a low effector to target cell ratio (e.g., 50:1). Also, the cell viability of differentiated HL-60 and two cell surface markers are monitored. The acceptable batch of HL-60 cells should have  $\leq 35\%$  apoptotic cell population characterized by Annexin V/propidium iodide staining,  $\geq 55\%$  CD35, and  $\leq 15\%$  CD71 expression. Baby rabbit complement is tested for non-specific killing and potency.

At present, a reference serum standard and a panel of proficiency sera should be prepared for an inter-laboratory comparison. After the initial inter-laboratory comparison using different qualified OPAs by each individual laboratory, we may be able to adopt a consensus OPA in the future.

**Steven Hildreth**, Sanofi Pasteur Inc, USA, Representative from IFPMA

A functional assay such as an OPA would facilitate the development of additional pneumococcal vaccines, which are needed. At Sanofi, the classical single-plex OPA is considered a labor-intensive, slow, and difficult assay. Nonetheless, the standardized OPA should be centered on the classical OPA before other OPA formats are considered. If an OPA is adopted as the standardized assay for pneumococcal antibody function, it should be validated and made to perform in a stable manner. To achieve these goals, assay components and processes must be controlled. The key components to be controlled include target bacteria (including viability, capsule, and standardized strains), phagocytes, complement, QC sera, and bacterial culture plates. There should also be a standardized source, handling criteria, and acceptance criteria for target bacteria and phagocytes. A reference serum should be made available. QC sera should represent different antibody levels. In addition, a method for in-house QC serum production should be developed. Data calculation should reflect the slopes and embrace multiple assay techniques.

**Daniel Sikkema**, Merck & Co. Inc., USA, Representative from IFPMA

Among the many vaccine biomarkers are toxin neutralization and serum bactericidal assay. Antibody quantity alone is insufficient as a correlate of immune protection. Sometimes other aspects such as antibody isotype, avidity, memory, or antibody function (opsonization) are needed. OPA has not yet been used as a vaccine biomarker because it is difficult to perform and has not been standardized.

Correlates of the protection provided by pneumococcal vaccines are unclear (i.e., the amount of protective antibody may vary) since there are many pneumococcal diseases (otitis, pneumonia, etc), two different target populations (children and the elderly), and different pneumococcal serotypes. To define the correlates of protection, a collaborative

study should be undertaken to investigate the timing of blood sampling, produce controls with different antibody levels, investigate more serotypes than the nine covered by the current pneumococcal vaccines, and develop shared methods.

**Manoj Kumar**, Serum Institute of India, Representative from Developing Country Vaccine Manufacturer's Network (DCVMN)

A functional assay such as an OPA is essential to the development of pneumococcal vaccines. There is a need to decide upon one of two different OPA formats existing currently (killing and flow-cytometric uptake methods), each with different advantages and disadvantages. In addition to defining phagocytes and target bacteria, developing country manufacturers need additional information about the equipments, culture media and maintenance of target bacteria, flow cytometer operation, and methods of data analysis. Thus, it is critically important to have a very detailed standard operating procedure available. Assay validation should encompass all the required elements including specificity, accuracy, linearity, robustness and precision. We favor efficient and simple options and would favor a multiplexed OPA.

**Helena Kayhty**, National Public Health Institute (KTL), Finland

The effect of HIV infection on the quality (potency) of pneumococcal antibodies was investigated using sera from children in South Africa (18). Antibody levels (measured by ELISA) and OPA titers were obtained on 30-60 persons per group. Opsonization titers for serotypes 6B, 19F, and 23F were determined using an uptake-type OPA (not killing-type OPA) because many of the infants were being treated with antibiotics. The two groups (HIV+ and HIV-) produced comparable levels of pneumococcal antibody. However, the HIV- group produced about 5-10 fold higher OPA titers than the HIV+ group. Also, the percentage of people with more than an OPA threshold (1:8) was higher in HIV- group than in HIV+ group. In addition, the HIV+ group required ~20 ng/ml of anti-6B antibody for 50% uptake while the HIV- group required only 2-3 ng/ml. These findings indicate that the HIV- individuals produce antibodies that are more effective than those produced by the HIV+ individuals. The findings also indicate that the functionality of pneumococcal antibodies should be monitored.

At the end of the presentation, a participant stated that similar findings were noted among HIV-infected adults. Another attendee indicated that a routine vaccine trial requires participants free of antibiotics.

**Milan Blake**, Center for Biologics Evaluation and Research, Food and Drug Administration (CBER/FDA), USA

By federal regulation, the U.S. FDA can accept a laboratory surrogate if a correlation between the laboratory surrogate and clinical effectiveness has been demonstrated. Abundant historical experiments have found that opsonization of pneumococci for phagocytes are the *in vivo* protective mechanism of anti-capsule antibodies. The OPA would be useful in assessing pneumococcal vaccines among the elderly since they are susceptible to pneumococcal infections despite having high levels of pneumococcal antibody as measured by ELISA (See Dr. Siber's presentation at

[http://www.fda.gov/ohrms/dockets/ac/05/slides/5-4188S2\\_5.PPT](http://www.fda.gov/ohrms/dockets/ac/05/slides/5-4188S2_5.PPT)). Pneumococcal antibodies from the elderly are about 10 fold less opsonic (per unit amount of antibody by ELISA) than antibodies from young children.

To consistently perform OPA, one should monitor target bacteria for capsule production, the status of phagocytes as determined by their surface phenotypes, and complement titer as determined by a CH50 (total hemolytic complement) assay. Rabbit sera may be useful as the source of complement for OPA. A previous study found that, although rabbit serum is more efficient in fixing C8 than human serum, both sera are equivalent in fixing C3 and C4 (19). To validate an OPA, its precision, accuracy, linearity, specificity, robustness, and assay stability should be determined. A multiplexed OPA would be desirable in view of the FDA's increasing analytical requirements. To assist with the development of OPA, the FDA is developing a reference serum. This reference serum will also replace 89-SF, which is currently used as the reference serum for pneumococcal antibody ELISA.

## **Designing an international collaborative study on standardization of OPA**

**Ian Feavers, NIBSC, UK**

Biologics, such as vaccines and biotherapeutics, are too complex to be characterized completely by physicochemical methods. The biologic process being assayed can be several steps removed from the end-point of the assay and the exact nature and mechanisms of action of biologics are not always known. Thus bioassays require the comparison of the test material with a standard or reference that has an arbitrarily assigned activity. WHO International Biological Standards are usually prepared and characterized through international collaborative studies (ICS). Recommendations for the preparation, characterization and establishment of international and other biological reference standards were recently updated. They include information on conducting an ICS and are published in WHO Technical Report Series (20).

An ICS is usually designed to demonstrate that a biological reference standard is fit for purpose. The aims of the study should be defined at the outset in consultation with WHO and potential participants. There is no generic design for an ICS but the study should be based on sound biological and statistical principles. The ICS should have a designated coordinator and biostatistician. As far as possible, the laboratories participating in any ICS should represent all WHO regions and user types (e.g., regulatory agencies and manufacturers). Clearly, the participating laboratories should be technically competent to carry out the assay(s) used in the study. A study protocol should be sent to all participants, all of whom should agree to complete the assay in a reasonable time (timelines should be agreed), in a safe and proper manner and not to use the materials for other purposes for which a Material Transfer Agreement (MTA) may be required.

For an ICS of the pneumococcal killing-type OPA (OPKA), a number of biological components have to be standardized including: the phagocytes; target pneumococci; testing serum; and complement source. Consideration also needs to be given to controls

and acceptance criteria for the assay. In designing such an ICS, the way in which data are collated, analyzed and eventually made publicly accessible should be taken into account. Since OPKA standardization will be a performance-based assay, a panel of reference sera will be required.

**Carl Frasch**, Frasch Biologics Consulting, USA

There are various publications relevant to OPA standardization and validation. Even though the standard OPA protocol has been well described in the literature, it should be further defined to include limits of detection (LOD) and limits of quantitation (LOQ) as well as information on standardized target bacteria and HL-60 cell phenotype characteristics. An improved calculation method is also needed. A curve-fitting program or calculation around two dilutions on either side of the 50% end point should be used to determine the opsonization index producing 50 % killing.

The standardization and validation of pneumococcal OPA requires a standardized source of HL-60 cells, standardized pneumococcal target strains, and calibration sera. The calibration sera are necessary for a performance-based standardization. A reference assay should be a killing-type OPA. A multiplexed assay is desirable and can be the primary assay. However, a different functional assay can be adopted as an operational assay after bridging the assay to a standard multiplexed killing assay. Sharing sera for comparison may not be sufficient but can be a first step towards standardization.

**Brian D. Plikaytis**, CDC, USA

To standardize OPA, it is crucial to have carefully designed inter-laboratory studies which can produce information on two important aspects of assay performance: repeatability (within-lab variability) and reproducibility (between-lab variability). To produce this information, the inter-laboratory study should employ an adequate number (e.g., 24) of specimens representing the full range of titers, should require all the laboratories to perform the same number of replicates (e.g., triplicates) for each specimen, should use a standardized data analysis procedure (e.g., a calculation method capable of providing endpoint determinations), and should require an OPA method(s) that is well characterized and validated within a laboratory. That is, various sources of variability (operator, day, instrument, protocol steps, etc.) of an OPA method should have been determined previously through an intra-laboratory assay validation.

Analysis of variance (ANOVA) can be used to measure repeatability and reproducibility in an inter-laboratory study. If it is used, the appropriateness of employing ANOVA should be established with descriptive statistics and graphical displays of results. The analysis must also consider the correlation among all specimens assayed within a particular laboratory. However, results will not reveal the true level of inter-laboratory agreement. Alternatively, one can use Mandel's two ( $h$  and  $k$ ) statistics (21, 22). His ' $k$ ' statistic compares repeatability among laboratories for a particular specimen and his ' $h$ ' statistic determines deviation of a laboratory from the overall average for a particular specimen.

To determine inter-laboratory agreement, conventional linear regression should not be used since it delivers biased results for the slope and intercept. Deming regression or regression that accounts for the variability in both laboratories (the  $x$  and the  $y$  variables) will yield a more accurate picture of inter-laboratory agreement. Pearson's correlation coefficient ( $r$ ) measures precision, not accuracy, so it can not be used to ascertain agreement between two laboratories. A statistic developed by Lin measures how well the data in a scatterplot conform to a  $45^\circ$  line (the coefficient of accuracy,  $C_a$ ). When coupled with the Pearson correlation coefficient, a combined measure is produced (the concordance correlation coefficient,  $r_c$ ) which pools both precision and accuracy into a single statistic (23).

## **Conclusions and recommendations**

There was agreement that a) the “gold standard” OPA assay should measure the opsonophagocytic killing, rather than uptake, of pneumococci and b) serum derived from adults immunized with 23-valent polysaccharide vaccine can be used to compare, control or qualify functional assays.

While a number of assays have been developed in academic, government and industrial laboratories, it is currently impossible to compare their performance. It was agreed that an iterative approach could be useful for the OPA standardization process. Consequently, a three-phased strategy is recommended for the standardization of the pneumococcal opsonophagocytic antibody assay.

### ***Phase one study***

Lyophilised sera available for immediate distribution from NIBSC will be used for an initial analysis of agreement between the OPA assays established in the Wyeth and GSK laboratories as well as with other laboratories currently running OPA assays.

- 24 sera (2 vials of each) will be selected by David Goldblatt, Ian Feavers and distributed (in blinded fashion) by NIBSC and will be made up of those with a range of titers as well as some blinded duplicates.
- Each serum will be run twice in each laboratory (on different days), as per individual protocols, and raw data for both runs submitted.
- OPA titres against 13 serotypes will be derived in laboratories running 13-valent OPA's routinely (GSK, Wyeth, Radboud University Nijmegen Medical Center, UAB) and at least 7 core serotypes will be tested in academic laboratories not running all 13 serotypes (KTL).
- Raw data will be supplied to a central point (UAB) for interpolation (according to a data transfer protocol drawn up by UAB), and interpolated data from individual laboratories will also be submitted.
- A statistical plan will be drawn up in advance to describe the data analysis (Brian Plikaytis /David Goldblatt) and the data will be sent from UAB to be evaluated by a statistician (Brian Plikaytis /David Goldblatt/Moon Nahm).

- Last data to come in by September 30<sup>th</sup> 2007 and analysis of data could be completed by the end of October 2007.
- Raw data submitted as part of the original OPA multilaboratory study will be reanalyzed using interpolation (Sandy Steiner and George Carlone).

If there proves to be good agreement between the GSK and Wyeth assays, values can be assigned to the serum panel, which can then be used for the subsequent standardization process. If there is no agreement, it will be necessary to examine the possible explanations before proceeding to the next phase, which will be designed to resolve the discrepancies between assays.

### ***Phase two study***

Although dependent on the outcome of phase one, plans for the second phase are expected to include reaching some agreement on the critical components of the OPA.

- It was agreed that the choice of bacterial strains, as well as growth conditions and time of harvesting were critical for the OPA and that a panel of standard strains would probably have to be established for phase two.
- The importance of the quality of effector cells was recognized. The acceptance criteria for HL-60 cells used in the assay should be harmonized (UAB will produce draft guidance). Together NIBSC and CBER will explore the feasibility of setting up a WHO master HL-60 cell bank specifically for the OPA assay.
- Guidance should be developed on the qualification of complement sources.
- A candidate reference serum, which was established by Professor Goldblatt, has been filled and lyophilized to meet WHO recommendations for international reference materials and is available at NIBSC. In addition, CBER has an ongoing project to replace the 89SF reference serum, which is currently used in the standardization of pneumococcal ELISAs. This will be dispensed aseptically without the addition of preservatives. Depending on timelines, this may also be available as a possible candidate reference serum for the OPA.

Agreement would also be needed for a WHO recommended OPA assay. The current OPA, as originally defined by Dr Romero-Steiner's publication and detailed on Dr Moon Nahm's website (<http://www.vaccine.uab.edu>), would serve as a useful basis for ongoing studies.

### ***Phase three study***

The third phase includes the evaluation of multiplex and other assays. It was agreed that some of this work could be done in parallel with phase two.

This three-phased approach recognizes a) the advanced level of assay development and validation already achieved by the pharmaceutical industry, b) the need to develop a WHO reference assay, c) the advantages of the standardization of assays on the basis of their ability to meet performance criteria rather than adherence to a prescriptive assay protocol, and d) the possibility of adopting appropriately validated alternative assay methodologies in the future.

### ***Additional activities***

Subgroups have been agreed working on the following subjects in order to harmonize the performance:

- 1) NIBSC (Ian Feavers/Roland Fleck), UAB (Moon Nahm) and CBER/FDA (Milan Blake) to work on the characterization of HL-60 cells, and explore the possibility of establishing a "WHO HL-60 cell bank" for use in OPA, with coordination from WHO. Focal point: Ian Feavers/Roland Fleck
- 2) UAB (Moon Nahm), Wyeth (Branda Hu) and GSK (Isabella Henckaerts) to draft a harmonized assay protocol or guidance for performing the assay and set up acceptance criteria for critical components. Focal point: UAB (Moon Nahm)
- 3) CDC (Brian Plikaytis) to work out a statistical plan/protocol, with help from NIBSC and CBER statisticians and DG. Focal point: CDC (Brian Plikaytis)
- 4) NIBSC (Ian Feavers) and CBER/FDA (Milan Blake) to explore the possibility of establishing an international reference serum for OPA, with coordination from WHO. Focal point: NIBSC (Ian Feavers)
- 5) Re-analysis of original multi-laboratory OPA study using standardised template Focal Point: CDC (Sandy Steiner and George Carlone)

Prof David Goldblatt, Dr Ian Feavers and Prof Moon Nahm have agreed to co-ordinate the studies overall.

The participants regarded this workshop a very timely effort in accelerating the standardization process of OPA and have shown strong willingness to collaborate in the subsequent studies.

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