Efficiency of a Pneumococcal Opsonophagocytic Killing Assay Improved by Multiplexing and by Coloring Colonies

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For evaluating pneumococcal vaccines, the opsonophagocytic killing assay (OPKA) is useful as a supplement to the pneumococcal antibody enzyme-linked immunosorbent assay (ELISA). However, evaluations of pneumococcal vaccines require the determination of antibody responses to 7 to 11 serotypes, and the OPKA is tedious to perform and requires more serum than the ELISA. Consequently, the OPKA is infrequently used for evaluating pneumococcal vaccines. To overcome these limitations, we have developed a simple multiplexed (double-serotype) OPKA by using antibiotic-resistant pneumococci for nine serotypes. Serotype 6B, 9V, 19A, and 23F strains were made streptomycin resistant, and serotype 4, 6A, 14, 18C, and 19F strains were made optochin resistant. The multiplexed OPKA was the same as the single-serotype OPKA except for two changes. First, the target bacteria were a mixture of one streptomycin-resistant strain and one optochin-resistant strain. Second, the surviving bacteria of each serotype were enumerated by plating on Todd-Hewitt agar plates with yeast extract and an agar overlay containing the appropriate antibiotics and 2,3,5-triphenyl tetrazolium chloride. The performance of the multiplexed OPKA was evaluated by analyzing 28 serum samples from adults immunized with a 23-valent polysaccharide vaccine by using the single-serotype OPKA and the multiplexed OPKA. The multiplexed OPKA was specific for the desired serotypes. The multiplexed and conventional OPKAs had comparable assay sensitivities and produced results that were highly correlated (r² values ranging from 0.92 to 0.98) for all nine serotypes. A simple modification of the conventional OPKA produces a multiplexed assay that greatly reduces effort, reagents, and the necessary amount of serum.

Streptococcus pneumoniae is an important pathogen for young children and older adults worldwide (8). An effective vaccine against pneumococci is highly desirable because conventional antibiotic treatment is becoming less effective due to the increasing rate of multidrug-resistant \textit{S. pneumoniae}. Even with effective antibiotic treatment, patients suffer from serious sequelae from invasive pneumococcal infections (31). Although a 23-valent polysaccharide (PS) vaccine has been available, it is not effective in young children (1, 23) and has a low efficacy in older adults (25). A recently introduced heptavalent conjugate vaccine is effective in young children (3, 22), but its serotype coverage is limited (15) and it is not effective in older adults (26). Further, there is a need to combine various vaccines to reduce the number of injections given to children (10). Thus, it is important to improve or modify the currently existing vaccines.

For evaluating new or modified pneumococcal vaccines, an enzyme-linked immunosorbent assay (ELISA) is commonly used to quantitate antibodies to serotype-specific \textit{S. pneumoniae} PS in pre- and postimmune sera. The ELISA for antibodies to pneumococcal PS has not been specific because the “purified” PS used for the ELISA is contaminated (28). Although the ELISA has been made more specific by neutralization of the interfering antibodies with cell wall PS (13, 19, 27) or irrelevant capsular PS (e.g., serotype 22F PS) (4), the ELISA may not be specific enough, since ELISA results do not perfectly correlate with the protective function of pneumococcal antibodies (6). Further, the ELISA may not differentiate nonprotective low-avidity antibodies from protective high-avidity antibodies.

To complement the pneumococcal antibody ELISA, the in vitro opsonophagocytic killing assay (OPKA) is often used (24). The in vitro OPKA for pneumococcal antibodies should predict immune protection and be a good surrogate assay for immune protection induced by the vaccine, since antibodies to pneumococcal capsular PS provide protection mainly by opsonophagocytosis. However, the in vitro OPKA is tedious to perform. Several improvements have been made to simplify the assay. For instance, HL-60, a pluripotent cell line, is used for effector cells to eliminate the need to isolate fresh granulocytes from human peripheral blood and at least some of the variability introduced (24). Despite improvements, the in vitro OPKA requires a large amount of serum and is not well suited for evaluating a large number of samples for antibody responses to the multiple (7 to 11) serotypes included in pneumococcal conjugate vaccines (16).

Nahm et al. recently reported the development of a double-serotype OPKA which yields the opsonization titers of two different serotypes in a single assay and reduces the amounts of reagents required (e.g., serum) by half (20). While this report demonstrated the feasibility of the concept, enumeration of the surviving bacterial colonies was still tedious, and it was unclear whether the double-serotype OPKA could be practical. To reduce the time and effort involved in counting colonies, we have made an additional improvement in the OPKA by incorporating a dye, 2,3,5-triphenyl tetrazolium chloride (TTC), into the agar plates. Live bacteria convert TTC to a product with a deep red color, and the colorized bacterial colonies are rapidly and easily enumerated (14). We describe here our...
development of the double-serotype OPKA for nine serotypes (serotypes 4, 6A, 6B, 9V, 14, 18C, 19A, 19F, and 23F) with all of the improvements listed above and its analytical characteristics.

**MATERIALS AND METHODS**

**Serum samples.** Adult volunteers were immunized with a 23-valent PS vaccine available from either Merck and Co. Inc., West Point, Pa., or Wyeth Lederle Vaccines, Pearl River, N.Y. Serum samples were obtained before and 1 month after vaccination. A human serum pool (pool 19H) was prepared by mixing sera from three individuals who received a pneumococcal PS vaccine 1 month before phlebotomy. The three individuals were selected based on the availability of sera. This pool was stored in aliquots at −20°C and used as a control in each OPKA. The pool and individual serum samples were incubated in a 35°C water bath for 30 min before OPKA.

**Production of antibiotic-resistant pneumococci of nine serotypes.** Pneumococci of serotypes 4, 6B, 9V, 14, 18C, 19A, 19F, and 23F (strains DS2382, DS2212, DS400, DS2214, GP116, DB18, DS2217, and DS2216) were originally obtained from G. Carlone, Centers for Disease Control and Prevention (Atlanta, Ga.). Pneumococci of serotype 6A (strain SP85) were obtained from D. Briles, Birmingham, Ala.

Optochin-resistant variants of DS2382, SP85, DS2214, GP116, and DS2217 were readily obtained by plating 10^8 CFU of the bacteria on a blood agar plate containing 5 mg of optochin (ethylhydrocupreine HCl; Sigma, St. Louis, Mo.)/liter. Each optochin plate yielded between 1 and 30 colonies. About 10 to 30 colonies were pooled together to establish an antibiotic-resistant subline.

To isolate strains resistant to streptomycin, DS2212, DS400, DB18, and DS2216 were plated at 10^7 CFU on a blood agar plate with 10 mg of streptomycin (Sigma)/liter. Several colonies from the plate were harvested, pooled, and expanded in Todd-Hewitt broth containing 0.5% yeast extract and 10 mg of streptomycin/liter, and the pneumococci were applied at 10^6 CFU to a blood agar plate containing 100 mg of streptomycin/liter. Variants of DS2212, DS400, DB18, and DS2216 that were resistant to 100 mg of streptomycin/liter were tested for sensitivity to optochin. Only variants sensitive to optochin were chosen for further studies.

Optochin-resistant variants of DS2382, SP85, DS2214, GP116, and DS2217 were labeled ORP_4, ORP_6A, ORP_14, ORP_18C, and ORP_19F, respectively. Streptomycin-resistant variants of DS2212, DS400, DB18, and DS2216 were labeled STRP_6B, STRP_9V, STRP_19A, and STRP_23F, respectively. These nine strains were grown in Todd-Hewitt broth containing 0.5% yeast extract to the log phase and divided into aliquots that were placed in 15% glycerol and frozen at −70°C for further use. The same frozen lot per strain was used for single-serotype and multiplexed (double-serotype) OPKAs throughout the entire investigation.

**Single-serotype and multiplexed OPKAs.** Single-serotype OPKA was performed as follows. Pneumococci that had been divided into aliquots and kept frozen at −70°C were used. HL-60 cells were differentiated into granulocytic cells by culturing them in RPMI 1640 with 10% fetal calf serum and 0.8% dimethylformamide (Fisher Scientific, Pittsburgh, Pa.) for 5 days. After differentiation, HL-60 cells were diluted to 10^7 cells/ml in Hank’s buffer supplemented with 0.1% gelatin and 10% fetal calf serum. To test the effect of HL-60 cells, fourfold fewer HL-60 cells were used in some experiments. Test samples (e.g., serum) were also diluted in the same buffer. Ten microliters of pneumococcal solution containing 1,000 CFU and 20 μl of test sample was placed in each well of a 96-well microtiter plate. After 30 min of incubation at room temperature, 40 μl of HL-60 cell suspension (4 × 10^5 cells per well) and 10 μl of baby rabbit complement (Pelfreeze, Brownlee, Wis.) were added to each well. The mixture was incubated for 1 h at 37°C with shaking. Five microliters of the reaction mixture was plated on Todd-Hewitt agar–yeast extract. After the fluid was absorbed into the agar, one plate was overlaid with Todd-Hewitt agar (0.75% yeast extract, 100 mg of streptomycin/liter, and TTC) and colonies counted on the streptomycin-containing plates were designated those of the streptomycin-resistant serotype.

**RESULTS**

**Development of an overlay method with TTC (colony colorization) and antibiotics.** To simplify the conversion from the conventional single-serotype OPKA to the multiplexed OPKA, we have developed an overlay method. In this method, we place a 0.75% agar overlay containing antibiotics and TTC over the conventional agar plate; the remainder of our assay is identical to the conventional single-serotype OPKA. To determine whether this method yields results that are comparable to those of the conventional method (i.e., colonies on a plate without an overlay), we examined the behavior of the bacterial culture with overlay agar containing various concentrations of antibiotics and TTC before we selected optimal concentrations for each. After testing optochin at 0.1 to 5 mg/liter and streptomycin from 10 to 300 mg/liter, we selected 0.5 mg of optochin/liter and 100 mg of streptomycin/liter as the optimal concentrations. At these concentrations, no inappropriate colonies grew. The antibiotic concentration could be either twofold lower or twofold higher than the chosen concentration without any effect on the number of the size of colonies.

Various TTC concentrations were also tested to find the optimal concentration (100 mg/liter) that turned the pneumococcal colonies deep red without altering the color of the agar plates or reducing the number of bacterial colonies compared to the results obtained with conventional ways of enumerating colonies (Fig. 1). One optimal concentration was applicable to all of the strains used in our study. Strain ORP_14 grew slowly, and the incubation time for the strain was increased from overnight to 2 days, but all other strains developed deep red colonies after overnight incubation. With TTC colorization, even very small colonies with a diameter of less than 1 mm were clearly visible (Fig. 1).

**Evaluation of different effector/target ratios.** Since the multiplexed OPKA method reduces the number of phagocytes per bacterium by twofold, we examined the effect of varying the ratio of phagocytes to bacteria for four different serotypes. We observed that the opsonization titers of sera were not significantly changed when the phagocyte/bacterium (effector/target) ratio was changed from 400:1 to 100:1 in the single-serotype OPKA for four serotypes, although the titers for serotypes 6B and 19A were lower than expected when the ratio was reduced to 40:1 (Fig. 2). Perhaps, at this assay condition, the number of functional phagocytes has become insufficient for phagocytosis. These results suggested that our assay conditions provide an excess number of phagocytes and enough for twofold more bacteria.

**Comparison of single-serotype and multiplexed OPKAs.** To directly compare the performance of the two OPKAs for all...
seven serotypes present in the conjugate vaccine (Prevnar) and two cross-reacting serotypes (serotypes 6A and 19A), we examined 28 serum samples with both the conventional single-serotype OPKA and the multiplexed OPKA. Twenty-three serum samples were obtained from adults immunized with the 23-valent PS vaccine, and 5 serum samples were obtained from preimmune adults (Fig. 3).

Both assay types had comparable precision, sensitivity, and specificity values. When a quality control sample was measured five times on 5 different days, the log-transformed values of its opsonization titer had standard deviations of 0.34 to 0.4 (i.e., about 2- to 2.5-fold variations) for two serotypes in both single-serotype and multiplexed assays. Both OPKAs had comparable sensitivities for all nine serotypes. In support of this conclusion, no serum sample had an undetectable opsonization titer in one OPKA and a detectable titer in the other OPKA, and a high correlation between the two test results was maintained even for sera with low titers. Several samples showed a high titer for one serotype and a very low or undetectable titer for other serotypes in the multiplexed OPKA for the nine serotypes. These observations suggested that the multiplexed OPKA was specific for the desired serotypes.

Both OPKAs produced results that were highly correlated ($r^2$ values ranging from 0.92 to 0.98) and yielded almost identical results for samples with high or low titers (Fig. 3). To compare the assay results, the number of samples that deviated more than twofold from the identity line was determined. We expected about 9 of 28 samples to differ by more than twofold from the identity line was determined. We expected about 9 of 28 samples to differ by more than twofold from the identity line was determined. We expected about 9 of 28 samples to differ by more than twofold from the identity line was determined. We expected about 9 of 28 samples to differ by more than twofold from the identity line was determined. We expected about 9 of 28 samples to differ by more than twofold from the identity line was determined. We expected about 9 of 28 samples to differ by more than twofold from the identity line was determined.

The overlay technique provides several advantages. It greatly simplifies the conversion of the conventional OPKA to the new, multiplexed OPKA because all of the necessary changes can be incorporated into the overlay agar. For instance, only conventional agar plates need to be prepared, and different antibiotics can be added to the overlay agar at the time of overlay. Also, the overlay technique reduces biohazards associated with OPKA, which uses live, potentially pathogenic bacteria, since the overlay shields the bacterial colonies. This feature may be relevant because the multiplexed OPKA uses antibiotic-resistant bacteria, and some assays use highly pathogenic bacteria, such as Neisseria meningitidis (17). The most important benefit of the incorporation of TTC into the overlay is that the bacterial colonies are colorized and more easily visualized. For unknown reasons, the bacterial colonies growing under the overlay are more intensely colored than those

FIG. 1. Microcolonies of S. pneumoniae serotype 6B on a Todd-Hewitt agar–yeast extract plate with an overlay containing TTC and antibiotic after 18 h of incubation. The average diameter of the microcolonies was about 0.3 mm, and the colonies were clearly red and distinct.

![Image](https://example.com/image1.png)

FIG. 2. Opsonization titers obtained in serum pool 19H with different effector/target ratios for serotypes 4, 6B, 19A, and 19F in the single-serotype OPKA. Opsonization titers did not change significantly at a 100:1 effector/target ratio but decreased at a 40:1 effector/target ratio for some serotypes.

DISCUSSION

Although it is very useful for vaccine evaluations, OPKA has been infrequently used because it is very tedious to perform. To partially overcome the limitations, Nahm et al. previously showed the feasibility of a multiplexed OPKA based on antibiotic-resistant pneumococci (20). We now describe a simple overlay technique that can be used to convert the conventional OPKA into a multiplexed OPKA. The technique also facilitates the automation of counting of bacterial colonies. Using this overlay technique, we have demonstrated that both multiplexed and conventional OPKAs produce comparable results for nine serotypes and that the technique is relevant for studying the immune response to a seven-serotype conjugate vaccine.

FIG. 3. Opsonization titers obtained in serum pool 19H with different effector/target ratios for serotypes 4, 6B, 19A, and 19F in the single-serotype OPKA. Opsonization titers did not change significantly at a 100:1 effector/target ratio but decreased at a 40:1 effector/target ratio for some serotypes.
FIG. 3. Opsonization titers obtained in the double-serotype method (y-axis) were compared with those obtained in the single-serotype method (x-axis) for serotype 4, 6A, 6B, 9V, 14, 18C, 19A, 19F, and 23F. Broken lines indicate twofold deviations from identity. Best fit lines were as follows: log(double)/H11005 = 0.98 log(single)/H11001 for serotype 4, log(double)/H11005 = 1.01 log(single)/H11001 for 6A, log(double)/H11005 = 0.94 log(single)/H11001 for 6B, log(double)/H11005 = 0.92 log(single)/H11001 for 9V, log(double)/H11005 = 0.99 log(single)/H11001 for 14, log(double)/H11005 = 0.96 log(single)/H11001 for 18C, log(double)/H11005 = 0.93 log(single)/H11001 for 19A, log(double)/H11005 = 0.94 log(single)/H11001 for 19F, and log(double)/H11005 = 0.93 log(single)/H11001 for 23F. r² values were 0.98, 0.96, 0.92, 0.93, 0.94, 0.97, 0.96, 0.97, and 0.98 for serotypes 4, 6A, 6B, 9V, 14, 18C, 19A, 19F, and 23F, respectively. One data point near the origin in each panel (marked with an arrow) represents serum samples with titers undetectable by both methods; the number in the circle represents the number of these samples. The titer sensitivity limit was 4.
Colorization of bacterial colonies facilitated counting of bacterial colonies in two ways. It increased the contrast between bacterial colonies and agar plates and made the bacterial colonies easily recognized by automated instruments. Second, TTC made even small bacterial colonies (less than 0.2 mm in diameter) detectable by a colony counter, which counts thousands of microcolonies in a single petri dish. Thus, we could routinely apply bacteria from 24 reaction wells into a single square petri dish and reduce the number of plates required to a manageable number, even for a large run of the OPKA. We found that the overlay technique with TTC has made the enumeration of bacterial colonies easy enough so that enumeration is no longer the limiting step in OPKAs or bactericidal assays.

TTC is one of the tetrazolium salts that have been used as indicators for cellular metabolism. TTC has been widely used in microbiology to rapidly determine drug susceptibilities of microorganisms (5, 29) and for rapid detection of bacteria in urine (7) or bacterial contamination in water (21). It has also been used to identify a pathological subspecies of Campylobacter (14) as well as to identify some Candida species (18). However, to our knowledge, TTC has never been used for OPKAs (or bactericidal assays) to enumerate bacterial colonies. TTC colorizes colonies of other species of bacteria (e.g., group B streptococci) and would be useful for OPKAs for other bacteria. In addition, 5-cyano-2,3-ditolyl tetrazolium chloride (12) may be interesting to investigate for its usefulness in OPKAs. It makes live bacteria fluorescent and would make even smaller bacterial colonies visible to automatic counters. Thus, this dye may make bacterial colony counting even more efficient by reducing the bacterial culture period and by accommodating samples from more than 24 OPKA wells in a single petri dish.

Although we could have increased the degree of multiplexing to many more serotypes, we analyzed only two serotypes at one time for several reasons. First, if we increased the degree of multiplexing, we might need bacterial strains that are resistant to clinically useful antibiotics, a situation which might increase the level of biohazard associated with the procedure. Second, further increases in multiplexing would change the effector/target ratio, and the consequences of altering this ratio would require investigation. Third, we might have to place pneumococci with similar serotypes (e.g., 6A and 6B) in a single well and have to consider the effect of two different target bacteria competing for cross-reactive antibodies. Last, the benefit of a double-serotype assay as described is sufficient because the throughput of the double-serotype assay is comparable to that of ELISA, the benchmark assay.

Because bacterial colony counting has been such a tedious step and readout for assays measuring the opsonic capacity of antibodies has been difficult, many opsonization assays have been developed with different readout methods. Some assay methods use a radiolabel (30), which is insensitive, inconvenient, and increasingly more difficult to use because of regulatory burdens. Another method uses flow cytometry (11, 16), which measures the phagocytosis of fluorescent bacteria by phagocytes and requires a flow cytometer, an expensive piece of specialized equipment, so that only certain laboratories can use this method. In addition, this method measures not opsonophagocytic killing but opsonophagocytosis and is less sensitive than the OPKA (16). Since the difficulties associated with counting of bacterial colonies have been overcome, bacterial colony counting should be considered the preferred readout method for opsonization assays.

Studies of pneumococcal vaccines require measuring protective functions of pneumococcal antibodies by many laboratories over a long period. Thus, there is a need for a standardized assay measuring the functions of pneumococcal antibodies. We believe that double-serotype OPKA may be the best candidate for this standardized assay. First, the assay is inherently attractive because it mimics the natural mechanism of antibody protection. Second, the double-serotype OPKA has become much easier to perform. Third, the double-serotype OPKA could be standardized more easily than other assays because investigators at the Centers for Disease Control and Prevention have largely standardized the single-serotype OPKA (24). Last, any laboratory can perform the double serotype OPKA because no special equipment is needed. In view of these factors, we are currently evaluating additional assay parameters (e.g., phase variability of bacterial strains and use of different phagocytic cell lines) (2, 9). In order to facilitate adoption of the standardized OPKA, we plan to make the assay protocol and reagents available as soon as the studies are complete.

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