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Colonies are clearly visible

New assay allows automated colony counting

S*treptococcus pneumoniae* is a major cause of pneumonia in young children and the elderly. New vaccines against this bacterium are needed because conventional antibiotic treatment is becoming less effective due to the increasing rate of multi drug-resistant *S. pneumoniae*. For evaluating novel or modified pneumococcal vaccines, an enzyme-linked immunosorbent assay (ELISA) is commonly used to quantify antibodies to serotype-specific *S. pneumoniae* but this is not specific because the "purified" protein used for the ELISA is contaminated¹. To complement the pneumococcal antibody ELISA, the *in vitro* opsonophagocytic-killing assay (OPKA) is often used² and a double-serotype OPKA, which substantially reduces the amounts of reagents has also been described³. However, evaluations of

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pneumococcal vaccines using the OPKA require the determination of antibody responses, which is commonly performed by enumerating surviving bacterial colonies. This can be extremely time consuming and as a consequence the OPKA, although it improves accuracy, is infrequently used for evaluating pneumococcal vaccines.

To overcome the colony counting difficulties involved in the OPKA, a dye (2,3,5-triphenyl tetrazolium chloride [TTC]) can be



Figure 1: A ProtoCOL automated colony counter

added to the agar plates⁴, so that live bacteria are coloured red, making them easier to visualise and count with a ProtoCOL automated colony counter (Figure 1).

Taking serum samples.

Adult volunteers were immunised with a vaccine from either Merck or Wyeth Lederle Vaccines. Serum samples were taken before and 1 month after vaccination. A human serum pool was prepared by mixing sera from three individuals who received a pneumococcal PS vaccine 1 month before phlebotomy, 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 (56°C, 30 min) before each OPKA.

Production of antibiotic-resistant pneumococci

Pneumococci of serotypes 4, 6B, 9V, 14, 18C,

19A, 19F, and 23F (strains DS2382, DS2212, DS400, DS2214, GP116, DB18, DS2217, and DS2216) were obtained from G. Carlone, Centers for Disease Control and Prevention (Atlanta, Ga,USA) and pneumococci of serotype 6A (strain SP85) were obtained from D. Briles, (Birmingham, Ala., USA). Antibiotic resistant variants of DS2382, SP85, DS2214, GP116, and DS2217 were produced by plating 108 CFU on a blood agar plate containing optochin (Sigma, St. Louis, Mo., USA) (5mg/l). Strains resistant to a second antibiotic were generated by plating strains DS2212, DS400, DB18, and DS2216 at 108 CFU on a blood agar plate with streptomycin (Sigma) (10mg/l). Each optochin plate yielded between 1 and 30 colonies which were pooled to establish an antibiotic-resistant subline and the optochin-resistant variants of DS2382, SP85, DS2214, GP116, and DS2217 were labelled ORPz4, ORPz6A, ORPz14, ORPz18C, and ORPz19F, respectively. Several colonies from the streptomycin plate were harvested, pooled, and grown in Todd-Hewitt broth containing yeast extract (0.5%) and streptomycin (10mg/l). The pneumococci were then plated at 108 CFU onto a blood agar plate containing streptomycin (100mg/l). Streptomycin-resistant variants of DS2212, DS400, DB18, and DS2216 were labelled STRPz6B, STRPz9V, STRPz19A, and STRPz23F, respectively.

Single-serotype and multiplexed OPKA's

Single-serotype OPKA was performed as follows. HL-60 cells were differentiated into granulocytic cells by culturing them in RPMI 1640 with 10% foetal calf serum and 0.8% dimethylformamide (Fisher Scientific, Pittsburgh, Pa, USA) for 5 days. After differentiation, HL-60 cells were diluted to 107 cells/ml in Hanks' buffer supplemented

with 0.1% gelatine and 10% foetal calf serum. A pneumococcal solution (10ml) containing 1,000 CFU and a human serum sample (20ml) were placed in each well of a 96-well plate and incubated (30min, room temperature). A HL-60 cell suspension of 105 cells (40ml) and baby rabbit complement (Pelfreeze, Browndeer, Wis., USA), (10ml) were added to each well. The mixture was incubated (1 h, 37°C, with shaking) and the reaction mix (5ml) was then plated on Todd-Hewitt agar–yeast extract. An overlay of Todd-Hewitt agar (0.75%) containing yeast extract (0.5%) TTC (Sigma) (100mg/l) and either optochin (at 0.1 to 5mg/l) or streptomycin (at 10 to 300mg/l) was poured on. The plates were incubated (37°C, 12 to 18 h), except serotype 14, (37°C, 36 h).

Surviving bacterial colonies of each serotype on the plates were counted using a ProtoCOL automated counter. The multiplexed OPKA was performed using the above assay protocol, except that the bacteria were an equal mixture of streptomycin-resistant (2x 10⁵ CFU/ml) and optochin-resistant (2 x10⁵ CFU/ml) pneumococcal strains. The bacterium pairs were STR-Pz6B and ORPz19F, STRPz9V and ORPz4, STRPz23F and ORPz18C, STRPz19A and ORPz6A, and ORP14 and STRP19A. After the assay, the reaction mix (5ml) was plated on two Todd-Hewitt agar–yeast extract plates. One plate was overlaid with Todd-Hewitt agar (0.75%) containing yeast extract (0.5%), optochin (0.5mg/l) and TTC (100mg/l). The other was overlaid with Todd-Hewitt agar containing yeast extract, streptomycin (100mg/l) and TTC (100mg/l).

Results

After testing optochin at 0.1 to 5mg/l and streptomycin at 10 to 300mg/l, optochin (0.5mg/l) and streptomycin (100mg/l) were found to be the optimal antibiotic concentrations. At these concentrations, no inappropriate

colonies grew and when the antibiotic concentration was either twofold lower or higher than the chosen concentration, this had no effect on the number or size of colonies. The optimal concentration of TTC is 100mg/l, this turns the pneumococcal colonies deep red without altering the agar plate colour or reducing the number of bacterial colonies. With TTC coloration, even very small colonies with a diameter of less than 1mm are clearly visible (Figure 2) and are easily enumerated with a ProtoCOL system.

Using this overlay technique, both multiplexed and conventional OPKA's produce comparable precision, sensitivity, and specificity results for nine serotypes. When a quality control sample was measured five times on five different days, the log-transformed values of its opsonisation titre had standard deviations of 0.34 to 0.4 (i.e., about 2- to 2.5-fold variations) for two serotypes in both single and multiplexed assays. Additionally, no serum sample had an undetectable opsonisation titre in one OPKA and a detectable titre in the other OPKA, and a high correlation between the two test results was maintained even for sera with low titres. Therefore, the multiplexed OPKA assay is relevant for studying the immune response to a seven-serotype conjugate pneumococcal vaccine.

Overcoming limitations

Although it is useful for vaccine evaluations, OPKA is infrequently used because it is tedious to perform. To overcome the limitations, a simple overlay technique can be used to convert the conventional OPKA into a multiplexed assay. The overlay technique 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. Also, with the overlay technique since the agar covers the bacterial colonies, this reduces any biohazards associat-

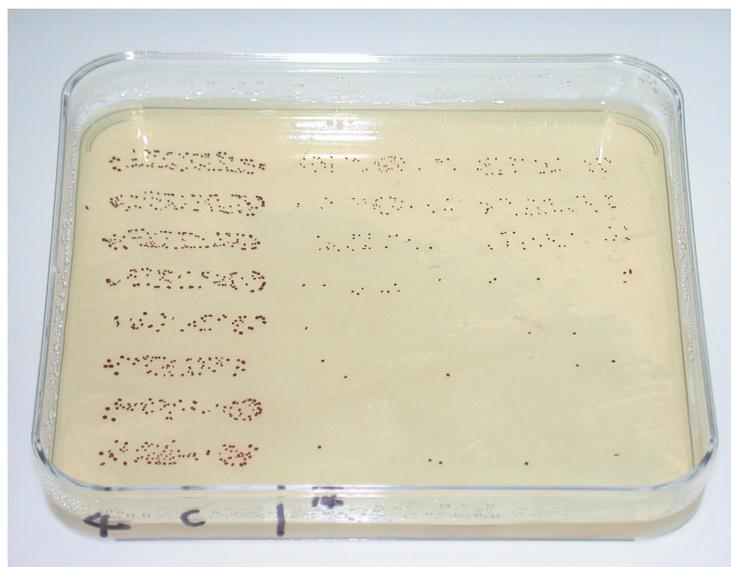


Figure 2: Microcolonies of *S. pneumoniae* serotype 6B on a Todd-Hewitt agar–yeast extract plate with an overlay containing TTC and antibiotic

ed with OPKA, which uses live pathogenic bacteria. The most important benefit of using an overlay is that a dye such as TTC can be added to the overlay, to produce coloured bacterial colonies, which are easier to visualise and count automatically.

Colouring bacterial colonies facilitates automated colony counting by increasing the contrast between bacterial colonies and agar plates, and allows the ProtoCOL system to detect bacterial colonies more easily. Additionally, TTC makes even small bacterial colonies (less than 0.2mm in diameter) detectable by a ProtoCOL colony counter, which can then count thousands of this type of microcolony in seconds. Therefore, it is simple to routinely take bacteria from 24 reaction wells into a single petri dish and reduce the number of plates required to a manageable number. The overlay technique with TTC makes enumeration of bacterial colonies with a ProtoCOL system easy enough so that colony counting is no longer the rate-limiting step in an OPKA. Thus, using a ProtoCOL makes the OPKA and coloured overlay method an excellent choice for evaluating new pneumococcal vaccines.

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By Kyung Hyo Kim, Jigui Yu, and Moon H. Nahm
Departments of Pathology and Microbiology,
University of Alabama at Birmingham, USA
enquiry number 08543