Elimination of False-Positive Results in a Luminex Assay for Pneumococcal Antibodies

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Received 13 August 2009/Returned for modification 4 September 2009/Accepted 11 November 2009

In our 14-valent Luminex assay for pneumococcal antibodies, we identified two groups of sera that caused false-positive results. The first group bound nonspecifically to the Luminex microspheres. The second group reacted specifically with bovine serum albumin (BSA). We describe here methods that eliminated the false-positive reactivity of both groups.

We previously described a multiplexed pneumococcal antibody assay based on the Luminex xMAP technology (11). Several other laboratories subsequently also described multiplexed Luminex assays for detecting these antibodies (1, 6, 15). In addition, the Luminex xMAP technology has been used for multiplexed assays to measure antibody responses to vaccines for other infectious diseases, including those caused by Neisseria meningitidis, Haemophilus influenzae type b, Bordetella pertussis, Clostridium tetani, Corynebacterium diphtheriae, Bacillus anthracis, and papilloma virus (3, 4, 10, 12, 13, 17). Waterboer et al. (18) documented an intrinsic problem with the use of the Luminex technology for serological assays. They found that some human sera bind directly to the carboxylated MicroPlex (formally MultiAnalyte) microspheres, causing a very high level of nonspecific background. These workers also found that SeroMAP microspheres, introduced by Luminex Corporation specifically for use in serological assays, reduced but did not eliminate the nonspecific-binding problem.

Using our original protocol (11) for the 14-valent pneumococcal antibody assay with lot B MicroPlex microspheres, we encountered serum samples with very high-level false-positive results that were near or above the analytical measurement range (AMR) of the assay (Table 1). Approximately 15 of every 1,000 sera tested for pneumococcal antibodies exhibited this behavior. We termed these samples “polyspecific,” although they did not react specifically to pneumococcal polysaccharides (PnPs). We tested a panel of 33 of these polyspecific sera and 1 control serum sample not showing polyspecific reactivity against an unconjugated MicroPlex microsphere, the MFI values for the SeroMAP microsphere suspended in B/S buffer, with median fluorescence intensity (MFI) values that ranged from 905 to 18,674. In contrast, the polyspecific sera, however, had background MFI values above 1,000. Two of the polyspecific sera, no. 3 and 23, had very high levels of nonspecific reactivity to the SeroMAP microspheres, with MFI values of 4,877 and 2,666, respectively.

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† Published ahead of print on 18 November 2009.
Nonspecific binding to uncoupled MicroPlex microspheres was completely eliminated by resuspending the uncoupled microspheres in StabliGuard (Fig. 1B). Compared to those for B/S buffer, the MFI values for the MicroPlex microspheres suspended in StabliGuard were reduced by an average of 99.7%. The MFI values for the 33 polyspecific sera against the uncoupled MicroPlex microsphere in StabliGuard ranged from 8 to 26. Except for the two sera (no. 3 and 23) whose MFI values were above 250, StabliGuard also eliminated the nonspecific binding of the 33 polyspecific sera to the SeroMAP microsphere.

We evaluated immunoglobulin-inhibiting reagent (IIR) (Bioreclamation, Inc., Westbury, NY), a reagent for removing heterophile antibodies, and polysorbate 20 (Tween 20; Sigma-Aldrich, St. Louis, MO) for their effects on nonspecific binding to MicroPlex and SeroMAP microspheres. IIR and Tween 20 were added separately to the sample diluent described above at concentrations of 400 μg/ml and 0.05%, respectively. The sample diluents with the two additives were compared to a sample diluent without an additive. Eight of the sera with high levels of nonspecific binding and the control serum sample, with no nonspecific binding (Fig. 1), were diluted 1:25 in each of the three sample diluents and tested in a single assay run against an uncoupled MicroPlex microsphere and an uncoupled SeroMAP microsphere suspended in either B/S buffer or StabliGuard. The results are shown in Fig. 2. As in Fig. 1A, the 7 polyspecific sera showed very high levels of nonspecific binding to the MicroPlex microsphere suspended in B/S buffer, with MFI values from 6,534 to 17,285 (Fig. 2A), and no nonspecific binding to the same microsphere suspended in StabliGuard (Fig. 2D). The MFI values for the 7 polyspecific sera with the SeroMAP microsphere in B/S buffer were 353 to 3,331 (Fig. 2A). Unlike those observed for the MicroPlex microsphere, however, the levels of nonspecific binding of the two high-binding-level sera (no. 3 and 23) to the SeroMAP microsphere were not completely blocked with StabliGuard and remained high, with MFI values of 1,249 and 916, respectively (Fig. 2D). Addition of Tween 20 to the sample diluent increased the nonspecific binding of the 7 polyspecific sera with both the MicroPlex and the SeroMAP microspheres suspended in B/S buffer by approximately 40% (Fig. 2B). Tween 20 also interfered with the blocking effect of StabliGuard. The levels of nonspecific binding of all the sera to both microspheres suspended in StabliGuard were higher with Tween 20 in the sample diluent than without Tween 20 (Fig. 2E). Addition of IIR to the sample diluent reduced the nonspecific binding of the polyspecific sera to the MicroPlex and SeroMAP microspheres suspended in B/S buffer by an average of 25% (Fig. 2C). IIR had no effect on the microspheres suspended in StabliGuard.

We modified our original 14-valent pneumococcal antibody assay.

### TABLE 1. IgG concentrations in serum before (protocol 1) and after (protocol 2) removal of nonspecific binding to microspheres

| Serum | Protocol | IgG concn (μg/ml) |
|-------|----------|-------------------|
|       |          | PnPs1 | PnPs3 | PnPs4 | PnPs5 | PnPs6B | PnPs7F | PnPs8 | PnPs9F | PnPs9V | PnPs12F | PnPs14 | PnPs18C | PnPs19F | PnPs23F |
| 3  | 1        | 11.44 | 14.40 | >11.61 | >80.03 | 5.94 | 37.37 | >32.83 | 4.56 | 25.25 | >18.03 | 17.41 | 19.89 | >27.77 | 19.90 |
| 2  |          | 1.42  | 7.03  | 0.28   | 2.21   | 0.22 | 1.31  | 0.33   | 0.35 | 0.61 | 0.46   | 1.78  | 0.88  | 1.78   | 0.12  |
| 9  | 1        | 19.52 | 15.89 | >11.61 | >80.09 | 10.62 | >37.00 | >32.83 | 11.45 | >25.25 | >18.03 | 38.63 | 39.44 | >27.77 | >28.78 |
| 2  |          | 1.16  | 0.36  | 0.29   | 2.06   | 0.31 | 1.16  | 1.00   | 0.53 | 0.61 | 0.84   | 8.37  | 2.91  | 0.35   | 0.76  |
| 23 | 1        | 11.44 | 14.46 | >11.61 | >80.09 | 5.94 | >37.00 | >32.83 | 7.56 | >25.25 | >18.03 | 19.41 | 19.89 | >27.77 | 19.90 |
| 3  |          | 2.00  | 4.83  | 0.31   | 2.78   | 0.22 | 1.56  | 0.38   | 0.57 | 1.02 | 0.67   | 2.05  | 1.23  | 0.17   | 0.32  |
| 28 | 1        | >20.40 | >24.74 | >11.61 | >80.09 | >48.48 | >37.00 | >32.83 | >20.71 | 25.25 | >18.03 | 17.88 | 16.85 | >27.77 | 23.02 |
| 2  |          | 0.33  | 0.32  | 0.14   | 1.69   | 0.16 | 0.81  | 0.14   | 0.16 | 0.48 | 0.24   | 0.26  | 4.49  | 3.02   | 4.57  |
| 34 | 1        | 0.90  | 3.01  | 0.17   | 1.74   | 0.32 | 1.83  | 2.20   | 0.18 | 0.76 | 0.44   | 0.44  | 0.80  | 0.41   | 0.57  |
| 2  |          | 0.97  | 1.83  | 0.53   | 1.45   | 0.45 | 1.27  | 3.17   | 0.10 | 0.41 | 0.35   | 6.12  | 0.32  | 0.13   | 0.30  |

FIG. 1. Nonspecific reactivity of human sera to Luminex microspheres. Shown are median fluorescence intensities for 33 polyspecific sera and a control serum sample reacted against unconjugated MicroPlex (clear bars) and SeroMAP (solid bars) microspheres. (A) Microspheres suspended in B/S buffer. (B) Microspheres suspended in StabliGuard.
to eliminate the false-positive assay results caused by the non-
specific reactivity of some sera to the microspheres. In our
original protocol, which we refer to as protocol 1, pneumococ-
cal polysaccharides (PnPs) were coupled with carboxylated
MicroPlex microspheres, washed in B/S buffer to block any
remaining activated carboxylated groups, and resuspended in
B/S buffer (11). In the revised protocol, which we refer to as
protocol 2, the MicroPlex microspheres were coupled with
PnPs, washed with B/S buffer, and resuspended in StabliGuard
rather than in B/S buffer. We also removed Tween 20 from the
sample diluent and the PBS wash buffer and added BCP dye to
the sample diluent. Both sample diluents contained 5
μg/ml each of C-Ps and 22F. Serum samples were diluted 1:25 in
protocol 1 and 1:101 in protocol 2. For both protocol 1 and
protocol 2, the assay was performed as described above.

Table 1 shows the Luminex pneumococcal IgG assay results
for four of the polyspecific sera and the control serum sample
(Fig. 1 and 2). The results obtained using protocol 1 are com-
pared to those obtained using protocol 2. The four sera with
very high levels of nonspecific reactivity to the MicroPlex mi-
crospheres produce high-level false-positive assay results for
all 14 PnPs serotypes when protocol 1 with microspheres sus-
pended in B/S buffer is used. The polyspecific reactivity is
eliminated in protocol 2 by using StabliGuard as the micro-
sphere diluent. The control serum sample (no. 34) that did not
react nonspecifically with the microspheres produced compa-
rable results with both protocols.

Using protocol 2, we encountered a subset of polyspecific
sera that did not react nonspecifically to the uncoupled micro-
spheres, and their false-positive reactivity was not affected by
StabliGuard. These sera appeared to react specifically to BSA.
These sera also reacted strongly to microspheres coated with
BSA. Protocol 2 was further modified to eliminate the B/S
wash step after the microspheres were coupled with PnPs,
which was found to be unnecessary with StabliGuard. In pro-
tocol 3, after PnPs were coupled with MicroPlex microspheres,
microspheres were washed in StabliGuard and resuspended in
StabliGuard. Otherwise, protocol 3 was identical to protocol 2.

Table 2 shows the polyspecific reactivities of two sera against
all 14 PnPs serotypes observed after MicroPlex microspheres

### TABLE 2. IgG concentrations in serum for 14 PnPs serotypes before (protocol 2) and after (protocol 3) removal of a BSA blocking step

| Serum | Protocol | PnPs1 | PnPs3 | PnPs4 | PnPs5 | PnPs6B | PnPs7F | PnPs8 | PnPs9N | PnPs9V | PnPs12F | PnPs14 | PnPs18C | PnPs19F | PnPs23F |
|-------|----------|-------|-------|-------|-------|--------|--------|-------|--------|--------|--------|-------|--------|--------|--------|
|       | 1        | 2     | 1.14  | 0.40  | 0.34  | 0.34   | 0.34   | 0.34  | 0.34   | 0.34   | 0.34   | 0.34  | 0.34   | 0.34   | 0.34   |
|       | 3        | 0.70  | 0.51  | 3.32  | 2.89  | 4.02   | 1.55   | 0.87  | 0.98   | 1.38   | 0.38   | 0.38  | 0.38   | 0.38   | 0.38   |
|       | 2        | 2     | 20.40 | 24.74 | 11.61 | 80.09  | 48.48  | 37.00 | 32.83  | 20.71  | 25.25  | 18.03 | 96.46  | 42.95  | 27.77  | 28.78  |
|       | 3        | 0.70  | 0.51  | 3.32  | 2.89  | 4.02   | 1.55   | 0.87  | 0.98   | 1.38   | 0.38   | 0.38  | 0.38   | 0.38   | 0.38   |
were blocked in B/S buffer prior to being resuspended in StabiGuard (protocol 2). The polyspecific reactivity was eliminated by eliminating the B/S blocking step in protocol 3.

The SeroMAP microspheres seem to us to be less desirable than the MicroPlex microspheres for use in Luminex-based serological assays. The SeroMAP microspheres also bind nonspecifically to some sera, although to a lesser degree than the MicroPlex microspheres (8, 18). Unlike what was observed for the MicroPlex microspheres, however, the nonspecific binding to the SeroMAP microspheres by two of the sera in this study could not be completely blocked with StabiGuard. Also, the SeroMAP microspheres have lower capacities for binding antigens, so the specific signals are lower than those observed with MicroPlex microspheres (18).

Waterboer et al. (18) approached the nonspecific-binding problem by adding additives to their serum preincubation buffer. They were able to suppress nonspecific binding to SeroMAP and MicroPlex microspheres by adding a combination of polyvinyl alcohol, polyvinylpyrrolidone, and Superchemiblock heterophile blocking agent (Millipore, Billerica, MA). The addition of additives to the preincubation buffer, however, reduced the specific signals by one-third (18). Super Chemiblock contains mouse immunoglobulins and is used to remove human anti-mouse antibodies (HAMA), heterophile antibodies, and rheumatoid factor. We evaluated a similar reagent, IIR. IIR contains purified immunoglobulins with high levels of affinity for anti-animal antibodies (5). In our hands, IIR reduced the nonspecific binding to unconjugated MicroPlex and SeroMAP microspheres by only about 25% (Fig. 1C). In addition, preabsorption of serum with heterophile blocking tubes (Scantibodies Laboratory, Inc., Santee, CA) had no effect. These reagents are effective in removing heterophile antibodies from other assay systems (5). Heterophile antibody interference in Luminex-based immunoassays for cytokines has been reported (2, 7). However, the reactivity of some human sera to the unconjugated Luminex MicroPlex and SeroMAP microspheres appears to us to be nonspecific binding of human IgG to the microspheres rather than a specific reaction of heterophile antibodies or other antibodies to antigenic determinants on the microspheres. The nonspecific binding was completely blocked with StabiGuard, a passive blocking agent. The above-mentioned additives to the sample diluent appear to function as competitive inhibitors of binding of IgG to the microspheres rather than as absorbents of specific antibodies.

Antibodies to BSA in serum from a 22-month-old child were reported to cause false-positive results in serological assays for human immunodeficiency virus type 1 and organisms causing other infectious diseases (19). Newell and coworkers also reported false-positive results in a commercial Luminex assay for antibodies to human leukocyte antigen (HLA) class I and class II antigens caused by antibodies to BSA used to block the microspheres (9). Anti-BSA antibodies have been reported to occur in 70% of children and 25% of adults (14). About 90% of the sera in this subset that showed false-positive reactivity in our pneumococcal antibody assay were from children 10 years of age or younger. These anti-BSA antibodies could possibly be absorbed out by addition of BSA to the sample diluent or microsphere diluents. However, the levels for the specific MFI signals were higher with protocol 3 after BSA was removed from the sample and microsphere diluents than with protocol 1. Also, StabiCoat Plus microarray stabilizer, a similar product from SurModics, Inc., but containing BSA, was less effective in eliminating nonspecific binding to the MicroPlex microspheres. Considering the high prevalence of antibodies to BSA in the population, we chose to eliminate the BSA blocking step and use BSA-free StabiGuard. The absence of BSA in the sample diluent allowed us to add an indicator dye (BCP) to the sample diluent to verify when a serum sample was added to the sample diluent (16). StabiGuard also allowed us to use a more concentrated serum dilution (1:25 dilution) for the assay. This improved the resolution of the assay at the low end of the AMR without incurring higher background levels.

In conclusion, blocking of the Luminex microspheres with StabiGuard and elimination of a BSA/PBS wash step after antigen coupling were simple, inexpensive, and effective methods for removing nonspecific reactivity in a Luminex-based serological assay for pneumococcal antibodies. Although we have not tested these procedures with other Luminex assays, the protocols described here might be applicable to other serological assays based on the Luminex xMAP technology.

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