Measuring Immunoglobulin G Antibodies to Tetanus Toxin, Diphtheria Toxin, and Pertussis Toxin with Single-Antigen Enzyme-Linked Immunosorbent Assays and a Bead-Based Multiplex Assay

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Bead-based assay systems offer the possibility of measuring several specific antibodies in one sample simultaneously. This study evaluated a vaccine panel of a multianalyte system that measures antibodies to tetanus toxin, diphtheria toxin, and pertussis toxin (PT) from Bordetella pertussis. The antibody concentrations of human immunoglobulin G (IgG) to PT, tetanus toxin, and diphtheria toxin were measured in 123 serum pairs (total of 246 sera) from a vaccine study. The multianalyte bead assay was compared to a standardized in-house IgG anti-PT enzyme-linked immunosorbent assay (ELISA) of the German reference laboratory for bordetellae, as well as to various commercially available ELISAs for anti-PT IgG, anti-tetanus IgG, and anti-diphtheria IgG. The results of the multiplex assay regarding the antibodies against diphtheria toxin compared favorably with a regression coefficient of 0.938 for values obtained with an ELISA from the same manufacturer used as a reference. Similarly, antibodies to tetanus toxin showed a correlation of 0.910 between the reference ELISA and the multianalyte assay. A correlation coefficient of 0.905 was found when an “in-house” IgG anti-PT and the multiplex assay were compared. Compared to single ELISA systems from two other manufacturers, the multiplex assay performed similarly well or better. The multianalyte assay system was a robust system with fast and accurate results, analyzing three parameters simultaneously in one sample. The system was well suited to quantitatively determine relevant vaccine induced antibodies compared to in-house and commercially available single-antigen ELISA systems.

MATERIALS AND METHODS

Serum samples. Anonymous surplus samples from a vaccine study were used. In the present study, 9- to 16-year-old adolescents with a low titer of IgG anti-PT, no history of pertussis immunization, and no history of pertussis received a single dose of an acellular combination vaccine that is licensed for adolescents and adults in Germany (Boostrix; GlaxoSmithKline Pharma GmbH & Co. KG, Munich, Germany) (3). Paired serum samples were taken before and 29 to 49 days after vaccination.

Methods. IgG antibodies to tetanus toxin were measured by three commercially available ELISAs and a multiplex bead assay: Serion ELISA classic tetanus (Virion/Serion, Würzburg, Germany), Virotech ELISA tetanus (Virotech, Rüsselsheim, Germany), and anti-tetanus toxoid ELISA Euroimmun (Euroimmun, Lübeck, Germany), and Serion Multianalyt diphtheria/tetanus/Bordetella pertussis toxin (Virion/Serion).

IgG antibodies against diphtheria toxin were measured by three commercially available ELISAs and the multiplex bead assay: Serion ELISA classic Diphtherie (Virion/Serion), Virotech ELISA Diphtherie (Virotech), Anti-Diphtherie-Toxoid ELISA Euroimmun (Euroimmun), and Serion Multianalyt diphtheria/tetanus/B. pertussis toxin (Virion/Serion).

IgG antibodies to pertussis toxin were measured by an in-house ELISA, two commercially available ELISAs, and the multiplex bead assay: in-house ELISA systems separately measuring IgG antibodies against PT, FHA, and pertactin (11); Serion ELISA classic B. pertussis toxin (Virion/Serion), Virotech ELISA pertussis toxin IgG (Virotech), and Serion Multianalyt diphtheria/tetanus/Bordetella pertussis toxin (Virion/Serion).

Reference methods. The following ELISA systems were defined as reference methods for the present study: IgG anti-tetanus toxin, Serion ELISA classic; IgG anti-diphtheria toxin, Serion ELISA classic; and IgG anti-PT, standardized in-house ELISA system (11). ELISAs for measuring antibodies against tetanus toxoid and diphtheria toxoid were previously validated against the Vero cell assay as the reference method (9), and for tetanus toxoid an in vivo mouse test was used as a reference method (8). No reference method exists for measuring antibodies to PT.

All ELISAs were run on a semiautomated washer, read on an E-max micro-
plate reader (Molecular Devices, Ismaning, Germany), and evaluated according to the manufacturer’s instructions. The in-house assay was evaluated by using a four-parameter logistic regression. Samples were diluted according to the manufacturers’ instructions. Samples with a value above the range of the standard curve were further diluted according to the manufacturers’ instructions.

**Multiplex bead assay.** The multiplex bead assay is based on a proprietary method patented in Germany in 1983 (7), and it has been used for the detection of antibodies to tetanus toxoid, diphtheria toxoid, and *B. pertussis* antigens (5, 6).

In brief, the method is based on microparticles of different sizes (e.g., 4.0 and 5.5 μm in diameter), which are labeled with a red fluorescent dye at different intensities. Target antigens, i.e., purified tetanus toxoid, purified diphtheria toxoid, and purified pertussis toxin, are coupled to the microparticles by covalent peptide bonding. The test also contains two different control particles that have a different fluorescent dye and serve as a function control for the test and for the cytometer. The test is done in microtiter filter plates. A serum dilution is mixed with the microparticles and incubated for 30 min. The cavities are emptied by vacuum again and washed four times. A second antibody to human IgG coupled to phycoerythrin is added, followed by incubation for another 30 min. The cavities are emptied by vacuum again and washed four times. Particles are resuspended by gentle mixing (5 min for 600 rpm on a horizontal shaker). The beads from every cavity are then measured with a flow cytometer. In our study, the test was run on an EPICS XL-MCL cytometer (Beckman-Coulter, Krefeld, Germany), and it was evaluated with assay-specific software (Serion Multianalyt evaluation software). The test format has also been validated for use with other flow cytometers, such as CyFlow (Partec Corp.) and FACSCalibur (BD Biosciences).

**Reference preparations.** The ELISAs used were based on the following reference preparations according to the information of the manufacturer.

**IgG anti-tetanus toxin.** All ELISAs and the multiplex bead assay refer to the World Health Organization (WHO) reference preparation, which is referred to differently by the various manufacturers (Multianalyt system, NIBSC code 76/589; Serion ELISA, 1.Intern.Standard, code TE-3; Virotech ELISA, WHO TE-3; Euroimmun ELISA, NIBSC code 76/589).

**IgG anti-diphtheria toxin.** The ELISAs and the multiplex bead assay refer to reference preparations by either WHO or by the Statens Seruminstitut, which are referred to differently by the manufacturers (Multianalyt system, NIBSC code 00/496; Serion ELISA, 1.Intern.Standard, Statens Seruminstitut, Copenhagen, Denmark; Virotech ELISA, NIBSC code 00/496; Euroimmun ELISA, NIBSC code 91/534.).

**IgG anti-PT in-house ELISA, multianalyte system, and Serion ELISA.** Values are measured in ELISA units (EU)/ml and refer to reference preparation 3 from the Laboratory of Pertussis, CBER, Food and Drug Administration (FDA), Bethesda, MD. The Virotech ELISA reports results as arbitrary units/ml (VE/ml), which should correlate to the CBER/FDA reference preparation.

**FIG. 1.** Scatter plot of values measured by the reference IgG anti-tetanus toxin ELISA versus those measured by the multianalyte assay (IU/ml).

**FIG. 2.** Plot of values in different ranges of IgG anti-tetanus toxin as measured by the multianalyte system (Multi) and two commercially available ELISAs compared to the reference ELISA. Symbols: ■, <1.0 IU/ml; ○, 1.0 to 5.0 IU/ml; ●, 5.0 to 10.0 IU/ml; △, >10 IU/ml. The concordance between the reference ELISA, two other ELISA systems, and the multianalyte system is demonstrated.

**FIG. 3.** Anti-tetanus toxin. A Bland-Altman plot of differences between reference ELISA and multianalyte system is shown.

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Lower levels of detection. Lower levels of detection were as follows: (i) for IgG anti-tetanus toxin, Serion ELISA (0.1 IU/ml), Serion Multianalyt (0.06 IU/ml), Virotech ELISA (0.1 IU/ml), and Euroimmun ELISA (0.01 IU/ml); (ii) for IgG anti-diphtheria toxin, Serion ELISA (0.05 IU/ml), Serion Multianalyt (0.025 IU/ml), Virotech ELISA (0.1 IU/ml), and Euroimmun ELISA (0.01 IU/ml); and (iii) for IgG anti-PT, in-house ELISA (2 EU/ml), Virion-Serion classic ELISA (5 EU/ml), Serion Multianalyt (4 EU/ml), and Virotech ELISA (1 VE/ml).

Range of primary standard curves. The ranges of primary standard curves were as follows: (i) for IgG anti-tetanus toxin, Serion ELISA (0.1 to 5.0 IU/ml), Serion Multianalyt (0.06 to 6.0 IU/ml), Virotech ELISA (0.1 to 5.0 IU/ml), and Euroimmun ELISA (0.01 to 10.0 IU/ml); (ii) for IgG anti-diphtheria toxin, Serion ELISA (0.05 to 2.0 IU/ml), Serion Multianalyt (0.025 to 6.0 IU/ml), Virotech ELISA (0.10 to 5.0 IU/ml), and Euroimmun ELISA (0.01 to 2.0 IU/ml); and (iii) for IgG anti-PT, in-house ELISA (2 to 200 EU/ml), Serion ELISA (5 to 600 EU/ml), Serion Multianalyt (4 to 500 EU/ml), and Virotech ELISA (not defined [0.6 to 51.5 VE/ml were used]).

Statistical evaluation. (i) Regression analysis. Scatter plots were constructed from primary data using the following adjustments. Values below the lower limit of detection were given 50% of the numeric value of the lower limit of detection. Values above the range of the standard curve (including secondary dilutions) were given the highest measurable value.

Scatter plots were made by using Sigma-Plot (SPSS, Jandel Scientific, Erkrath, Germany) including a linear regression line. In addition, the 95% prediction intervals were also plotted. For some comparisons, scatter plots from all results were constructed by using the quantitative values of all ELISAs and the multianalyte system with different ranges. These ranges were as follows: for IgG anti-tetanus toxin <1.0 IU/ml, 1.0 to 2.0 IU/ml, 2.0 to 10.0 IU/ml, and >10.0 IU/ml; for IgG anti-diphtheria toxin, <0.1 IU/ml, 0.1 to 1.0 IU/ml, 1.0 to 2.0 IU/ml, and >2.0 IU/ml; and for IgG anti-PT, <0.5 EU/ml, 0.5 to 100 EU/ml, and >100 EU/ml. Bland-Altman plots were constructed by using GraphPad software (GraphPad Software, San Diego, CA).

(ii) Relative concordance of ranges. In order to determine the relative concordance of ranges, it is important to compare ranges of antibodies, since these may be clinically relevant to the interpretation of results. The following ranges were defined and compared. For IgG anti-tetanus toxin, the values were <1.0 IU/ml, 1.0 to 10.0 IU/ml, and >10.0 IU/ml. A level of >1.0 IU/ml is indicative of immune protection. For IgG anti-diphtheria toxin, the values were <0.1 IU/ml, 0.1 to 2.0 IU/ml, and >2.0 IU/ml. A level of >0.1 IU/ml is regarded as the threshold for immune protection. For IgG anti-PT, the values were <0.5 EU/ml, 0.5 to 100 EU/ml, and >100 EU/ml. Since no accepted levels of protection for antibodies for pertussis exist, a level of <0.5 EU/ml was regarded as probably indicative of no recent contact, a level between 50 EU/ml and 100 EU/ml was regarded as indeterminate, and a level of >100 EU/ml was regarded as an indicator of recent contact (1).

### RESULTS

Antibodies to tetanus toxin. The concentrations of antibodies measured by the multiplex assay were plotted in scatter plots against the ELISA values (Fig. 1). A regression coefficient of 0.910 was found compared to the reference ELISA. The y intercept of the curve was −0.12 IU/ml, and the slope was 1.17. Compared to the reference ELISA, the regression coefficients of the other ELISA systems were 0.935 (Euroimmun) and 0.832 (Virotech), respectively. The intercepts of the other ELISA systems were −0.31 (Euroimmun) and −0.32 (Virotech), respectively, and the slopes of the regression curves were 1.21 (Euroimmun) and 0.964 (Virotech), respectively. Another scatter plot (Fig. 2) shows the values of the multianalyte system and two other commercial ELISAs compared to the reference ELISA. Values are displayed in four different ranges: <1.0 IU/ml, 1.0 to 5.0 IU/ml, 5.0 to 10.0 IU/ml, and >10 IU/ml. It is obvious that the overlapping of ranges was similar between all systems compared. A Bland-Altman plot of the bias between the reference ELISA and the multianalyte system (Fig. 3) shows no relevant bias between both methods.

Table 1 compares the concordance in which three diagnostically relevant cutoffs were used. These were <0.1 IU/ml, 0.1 to 1.0 IU/ml, 1.0 to 10.0 IU/ml, and >10 IU/ml. The systems agreed by 96, 72, and 94%, respectively (Tables 1 to 3). In the medium range (1.0 to 10 IU/ml) the multiplex assay showed higher values (19% were >10 IU/ml). When the multianalyte

### Table 1. Concordance of ranges for IgG anti-tetanus toxin antibodies (reference ELISA versus multianalyte assay)

<table>
<thead>
<tr>
<th>Virion ELISA result (IU/ml)</th>
<th>n</th>
<th>% Specimens with a multianalyte assay result of:</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;0.1</td>
<td>13</td>
<td>100</td>
</tr>
<tr>
<td>0.1–2.0</td>
<td>129</td>
<td>11</td>
</tr>
<tr>
<td>&gt;2.0</td>
<td>107</td>
<td>0</td>
</tr>
<tr>
<td>1.0–10.0</td>
<td>162</td>
<td>9</td>
</tr>
<tr>
<td>&gt;10.0</td>
<td>61</td>
<td>0</td>
</tr>
</tbody>
</table>

* No. of specimens.

### Table 2. Concordance of ranges for IgG anti-diphtheria toxin antibodies (reference ELISA versus multianalyte assay)

<table>
<thead>
<tr>
<th>Virion ELISA result (IU/ml)</th>
<th>n</th>
<th>% Specimens with a multianalyte assay result of:</th>
</tr>
</thead>
<tbody>
<tr>
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<tr>
<td>&gt;10.0</td>
<td>61</td>
<td>0</td>
</tr>
</tbody>
</table>

* No. of specimens.
assay was compared to the Virotech assay, both tests agreed by 96, 74, and 63%, and with the Euroimmun ELISA the concordance was 100, 85, and 87%.

**Antibodies to diphtheria toxin.** The concentrations of antibodies measured by the multiplex assay were plotted in scatter plots against the ELISA values (Fig. 4). A linear regression of 0.938 was found compared to the reference ELISA. The y intercept of the curve was −0.11 IU/ml, and the slope was 1.19. Compared to the reference ELISA, the regression coefficients of the other ELISA systems were 0.934 (Euroimmun) and 0.916 (Virotech), respectively. The intercepts of the other ELISA systems were −0.67 (Euroimmun) and −0.19 (Virotech), respectively, and the slopes of the regression curves were 1.14 (Euroimmun) and 0.963 (Virotech), respectively.

Figure 5 shows the values of the multiplex system and two other commercial ELISAs compared to the reference ELISA. Values are displayed in four different ranges: <0.1 IU/ml, 0.1 to 1.0 IU/ml, 1.0 to 2.0 IU/ml, and >2.0 IU/ml. It is obvious that the overlapping of ranges was comparable between the systems. The Virotech ELISA had a lower level of detection of 0.1 IU/ml. A Bland-Altman plot of the bias between the reference ELISA and the multiplex system (Fig. 6) shows no relevant bias between the two methods.

Table 2 compares the diagnostically relevant cutoffs. For anti-diphtheria toxin, these were <0.1 IU/ml, 0.1 to 2.0 IU/ml, and >2 IU/ml. The systems agreed by 100, 83, and 94%, respectively. When the multiplex assay was compared to the Virotech assay, both tests agreed by 92, 68, and 75%, and with the Euroimmun ELISA the concordance was 92, 60, and 5%.

**Antibodies to pertussis antigens (IgG anti-PT).** The concentrations of antibodies measured by the multiplex assay were plotted in scatter plots against the ELISA values (Fig. 7). A linear regression of 0.905 was found compared to the reference ELISA. The y intercept of the curve was 0.68 EU/ml, and the slope was 0.81. Compared to the reference ELISA, the regression coefficients of the other ELISA systems were 0.869.
and 0.816 (Virotech), respectively. The intercepts of the other ELISA systems were 0.40 (Virion) and 0.36 (Virotech), respectively, and the slopes of the regression curves were 0.87 (Virion) and 0.42 (Virotech), respectively.

Figure 8 shows the values of the multianalyte system and two other commercial ELISAs compared to the reference ELISA. Values are displayed in three different ranges: <50 EU/ml, 50 to 100 EU/ml, and >100 EU/ml. The Virotech ELISA uses a different unitage, which is shown on the right y axis of the figure. A Bland-Altman plot of the bias between the reference ELISA and the multianalyte system (Fig. 9) showed that the reference ELISA measured systematically lower levels, when sera contained medium to high concentrations of anti-PT.

Table 3 also displays possible diagnostically relevant cutoffs for anti-PT. These were <50 EU/ml, 50 to 100 EU/ml, and >100 EU/ml. The systems agreed by 82, 9, and 98% (Tables 1 to 3). As also shown in Table 3, the multiplex assay identified 91% of the sera which were between 50 and 100 EU/ml in the reference assay as being >100 EU/ml. When we compared the multiplex assay to the Serion ELISA, both tests agreed by 87, 30, and 94%, respectively. The Virotech ELISA measures in different units, so a comparison could not be made.

All methods showed a 100% agreement for the serum pairs.

The increase in signal after vaccination was significant in all ELISAs and the multianalyte method.

DISCUSSION

Antibodies to tetanus toxin as a result of immunization have been measured primarily by mouse neutralization tests. ELISA formats have reproducibly yielded comparable results, and they are now widely used to measure the vaccination response (8). Primarily derived from the mouse model, protective cutoffs for antibodies have been established, and these are now widely used in seroepidemiological studies to measure the population immunity to this antigen.

Similarly, antibodies to diphtheria toxin were primarily measured with an animal system, i.e., a guinea pig intradermal test. In addition to ELISA systems, which are used for routine purposes, the antibody response to vaccination can also reliably be measured by a Vero cell assay (9). Similar to tetanus toxin, protective thresholds have been defined that should guarantee protection against the toxin with a high level of probability.

ELISA systems that measure antibodies to tetanus and diphtheria toxin are commercially available; these assays have to be licensed by the FDA in the United States, and they must comply with the in vitro diagnostic directive of the European Union. Methods for measuring antibodies to B. pertussis antigens such as PT or FHA are less well standardized. Commercially available tests in the European Union show variable results (4), and no FDA-approved test is available in the United States. In contrast to antibodies to tetanus or diphtheria toxin, no serological correlate for protective immunity exists (1, 2).

Although ELISA systems are well characterized and rather reliable, they require an aliquot of a patient sample for every test, and the cost for labor and materials increases with the number of analyses.

A microsphere-based flow cytometry technique circumvents

FIG. 8. Plot of values in different ranges of IgG anti-PT as measured by the multianalyte system (Multi) and two commercially available ELISAs compared to the reference ELISA. Symbols: ■, <50 EU/ml; ○, 50 to 100 EU/ml; △, >100 EU/ml.

FIG. 9. Anti-PT. A Bland-Altman plot of differences between reference ELISA and multianalyte system is shown.
these issues by analyzing several parameters in one sample simultaneously (5, 6, 7, 10). Flow cytometers are widely used in laboratories, and thus this assay system can use existing equipment. In a multiplex bead assay, serum antibodies are in closer contact with the antigens bound to the beads, making the test faster. Compared to single ELISAs, the overall turnover time is much shorter and less sample volume is needed. The simultaneous analysis of all samples allows for the simultaneous validation of results.

Among various other applications of this technique, methods have been described to measure antibodies to diphtheria and tetanus toxin and *Haemophilus influenzae* type b capsular polysaccharide (5) and to PT and FHA of *B. pertussis* (6). These studies used other antigen sources and compositions and also different ELISA comparators. Given these caveats, the multiplex bead assay compared well in our study and in a previous study (5) with single ELISAs for measuring antibodies to tetanus and diphtheria toxoids. The multiplex assay used previously (6) was meant as a diagnostic tool and thus measured IgG and IgA antibodies to PT and FHA. With respect to IgG anti-PT, the concordance between the ELISA and the multianalyte system was 82% for concentrations of <50 EU/ml, which was comparable (6). Sera with high levels of anti-PT were measured mostly lower with the reference ELISA. However, the multianalyte system detected all titer increases after vaccination that were detected by single-antigen ELISAs and thus seems well suited for measuring vaccine responses.

In conclusion, the multiplex assay tested here was a robust system, providing rapid and accurate results. It showed good concordance with various single-antigen ELISAs and performed well in measuring the immune response after vaccination. It thus offers an alternative to ELISA systems for measuring vaccine responses in routine and research laboratories.

REFERENCES