Diphtheria is caused by toxigenic strains of *Corynebacterium diphtheriae* and is almost entirely preventable through a regular immunization schedule (9). Since routine immunization against diphtheria has become standard practice in industrialized nations, clinical cases are rare; however, it is still a problem in many developing countries, such as the new nations formed from the breakup of the former Soviet Union (29), as well as in people without adequate immunity.

As well as determining the rates of immunity within broad populations and the immune status of at-risk individuals, accurate measurements of anti-diphtheria toxoid IgG levels are important in assessing the response to vaccination and the efficacy of an immunization schedule (13, 16, 31) and in evaluating individuals for potential immunodeficiency disorders (5). Vaccination response studies are part of the clinical testing recommended for the diagnosis of primary immunodeficiency (2, 5).

For determination of anti-diphtheria toxoid IgG antibodies, the *in vivo* neutralization test (NT) and *in vitro* Vero cell assay (VCA) are considered the gold-standard methods (10, 18). However, enzyme-linked immunosorbent assay (ELISA)-based methodologies offer a simpler, safer technique, as live toxin is not required and an ELISA is a more rapid and less expensive technique than a neutralization test. It is often preferred by clinical laboratories to measure anti-diphtheria toxoid IgG levels.

Standardization of anti-diphtheria toxoid IgG tests has been facilitated by the availability of reference material, allowing results to be given in international units (IU). The international standard for anti-diphtheria toxoid IgG (NIBSC 00/496) was assigned a value of 0.8 IU/ml by comparison to the British standard for equine anti-diphtheria toxin (NIBSC 66/153) in the mouse *in vivo* neutralization test. The World Health Organization (WHO) states that a specific IgG concentration of 0.1 IU/ml is usually considered protective by standard ELISA (27). Many studies have incorporated the WHO guidelines and interpret results <0.01 IU/ml as not protective against infection, 0.01 to 0.09 IU/ml as basic protective levels, and >0.1 IU/ml as protective levels (4, 5, 7, 8).

The titer of anti-diphtheria IgG antibodies in patients with inadequate immunity or an immunodeficiency disease can be very low, often <0.1 IU/ml. To be able to accurately and reliably measure low-level titers, ELISAs need to be extremely sensitive. This report compares the sensitivities of five commercially available anti-diphtheria toxoid IgG ELISA kits and suggests that the manufacture-dependent differences in preparation of these assays may affect the clinical interpretation of data.
The response to diphtheria vaccination was assessed using 12 pre- and post-vaccination sample pairs, assayed over two consecutive days. The samples were therefore not included in Fig. 1. All five ELISAs identified a number of samples with titers of anti-diphtheria IgG antibodies below the WHO-recommended level of protection (0.1 IU/ml): Euroimmun, 37/56 samples (66%); Scimedx, 3/56 samples (5.3%); Serion, 21/56 samples (38%); BS, 26/56 samples (46.4%); and Virotech, 9/56 samples (17%).

Figure 1 also shows that there were no statistical differences between the Euroimmun and BS assays (Wilcoxon test, \(P = 0.375\)) and the Scimedx, Serion, and Virotech assays (Friedman’s test, \(P = 0.295\)) but there was a significant difference between the two groups of data (Wilcoxon test, \(P < 0.0001\)). Figure 1 also suggests that there was not an even distribution of sample values around the median in the Euroimmun and Scimedx assays. Using the samples that were quantifiable in all

![Graph](image-url)
fifteen assays, there was good agreement in regard to positivity (>0.1 IU/ml) and negativity (<0.1 IU/ml) between the five assays. A comparison of relative sensitivity between the five assays was 88.6% to 100%, and relative specificity between four of the assays was 72.7% to 90.5% with the exception of the Scimedx assay (45% to 52.6%).

To assess the accuracy of calibration, the recoveries of serially diluted NIBSC 00/496 reference material with known titers, read from the respective calibration material, were assessed and expressed as a percentage of the target value (Table 2). To allow fair comparison across all five assays, recovery across a measuring range of 0.11 to 3 IU/ml was compared. Two of the five assays (BS and Virotech) gave consistent and accurate recoveries of the reference material across the range tested, with percent CV <10%. When recoveries around the protective cutoff of 0.1 IU/ml were assessed, only the Scimedx (95.1%), BS (97.1%), and Virotech (91.5%) assays showed recoveries within 10% of the target value. When the diluted reference material at target values of 0.037 and 0.012 IU/ml were run on the two assays designed to measure that low, the values were 108.1% and 130.3%, respectively, in the BS assay, but were undetectable in the Euroimmun assay.

Levels of anti-diphtheria toxin IgG antibodies were determined in 12 paired pre- and postvaccination samples (Table 3). It was not possible to obtain quantitative values for all prevaccination samples by all five assays, as some samples were below the limit of detection in certain assays.

It has been proposed that a 4-fold or greater increase in the specific antibody titer is a clinically significant response to diphtheria vaccine (16). It has also been suggested that a nonresponder would exhibit a <2.6-fold increase in antibody titers after vaccination or antibody titers less than the protective titer postvaccination (17, 27). The titers of anti-diphtheria IgG antibodies were <0.1 IU/ml for prevaccination samples 9 and 21 in the Euroimmun assay and >0.1 IU/ml for prevaccination samples 10, 14, 17, 19, and 26 in the Scimedx assay, which were out of consensus with titers measured in the other four assays. With the anti-diphtheria IgG titers recorded in samples 10, 16, and 19 in the Scimedx assay, a 4-fold increase in titer was not achieved postvaccination but the prevaccination levels indicated titers >0.1 IU/ml.

For both the Serion and Virotech assays, it was difficult to accurately measure an increase in titer for samples 10, 14, 17, and 19, as well as for sample 22 in the Serion assay and sample 26 in the Virotech assay, as the prevaccination anti-diphtheria IgG antibody titers were lower than the measuring range of the kit. Fourfold increases in antibody titer could not be measured in any assay for sample 16; however, a prevaccination level of >0.1 IU/ml and no 4-fold increase in titer were measured in sample 26 in the Serion assay, but the postvaccination level was >0.1 IU/ml. The value measured for prevaccination sample 22 in the Virotech assay was >0.1 IU/ml, which was out of consensus with values obtained in the other four assays, which either recorded values lower than the measuring range or falling in the range but were undetectable in the Euroimmun assay.

In the BS assay, 4-fold increases in anti-diphtheria IgG titers

### Table 2. Mean percentage recovery from three repeat assays of the NIBSC 00/496 standard in five commercial ELISAsa

| Target (IU/ml) | Recovery (%) | Euroimmun | Scimedx | Serion | BS | Virotech |
|---------------|-------------|-----------|---------|--------|----|---------|
| 3.00          |             | 45.5      | 149.3   | NA     | 85.3| 107.6   |
| 1.00          |             | 98.0      | 127.7   | 104.9  | 89.7| 114.1   |
| 0.33          |             | 83.6      | 86.1    | 94.5   | 94.8| 100.2   |
| 0.11          |             | 37.5      | 95.1    | 82.3   | 97.1| 91.5    |
| 0.037         |             | 0.00      | N/A     | N/A    | 108.1| N/A     |
| 0.012         |             | 0.00      | N/A     | N/A    | 130.3| N/A     |
| Mean          |             | 66.1      | 114.7   | 93.9   | 91.7| 103.3   |
| CV            |             | 44.3      | 25.4    | 12.1   | 5.8| 9.4     |

a To calculate values, values (IU/ml) for serially diluted NIBSC 00/496 were obtained from a calibration curve and compared to the expected value according to the following equation: (obtained NIBSC value/expected NIBSC value) x 100. NA, not applicable.

b Mean value and CV are for the recovery of samples of 0.11 to 3.00 IU/ml.

c The measuring range of this kit was 0.1 to 5 IU/ml (see Materials and Methods).

d The measuring range of this kit was 0.05 to 2.0 IU/ml (see Materials and Methods).

### Table 3. Pre- and postvaccination data from five commercial ELISAsa

| Sample ID | Pre | Post | Fold | Pre | Post | Fold | Pre | Post | Fold | Pre | Post | Fold |
|-----------|-----|------|------|-----|------|------|-----|------|------|-----|------|------|
| 9         | 0.093| 1.089| 11.7 | 0.186| 1.547| 8.3  | 0.113| 1.286| 11.4 | 0.129| 0.914| 7.1  |
| 10        | 0.018| 0.174| 9.7  | 0.103| 0.333| 3.2  | <0.05| 0.031| 3.0  | 0.046| 0.218| 4.8  |
| 14        | 0.018| 0.822| 46.3 | 0.103| 1.257| 12.2 | <0.05| 0.861| 17.1 | 0.049| 0.838| 17.1 |
| 15        | 0.232| 1.271| 5.5  | 0.246| 2.445| 10.0 | 0.458| 2.000| 4.8  | 0.431| 1.458| 3.4  |
| 16        | 0.077| 0.494| 6.4  | 0.175| 0.560| 3.2  | 0.220| 0.608| 2.8  | 0.191| 0.417| 2.2  |
| 17        | 0.045| 0.884| 19.4 | 0.138| 1.186| 8.6  | <0.05| 0.776| 8.3  | 0.065| 0.567| 8.8  |
| 19        | 0.011| 0.146| 13.0 | 0.110| 0.228| 2.1  | <0.05| 0.027| 6.0  | 0.099| 0.266| 6.0  |
| 20        | 0.160| 0.495| 3.1  | 0.622| 1.034| 1.7  | 0.630| 0.930| 1.5  | 0.377| 0.614| 1.6  |
| 21        | 0.069| 1.233| 17.8 | 0.137| 3.806| 27.7 | 0.265| >2.000| 12.3 | 0.216| 1.476| 6.8  |
| 22        | <0.01| 0.568| <1.1  | 0.097| <0.5| 0.557 | <0.05| 0.025| 18.2 | 0.154| 0.765| 5.0  |
| 26        | 0.054| 0.308| 5.7  | 0.149| 0.336| 2.3  | 0.061| 0.233| 3.8  | 0.065| 0.178| 2.7  |
| 28        | 0.014| 0.018| 1.3  | <0.1| <0.1| <0.1  | <0.05| 0.021| 1.7  | <0.1| <0.1| <0.1  |
| Mean      | 0.072| 0.625|      | 0.197| 1.240|      | 0.291| 0.585|      | 0.138| 0.620|      |
| SD        | 0.069| 0.431|      | 0.156| 1.068|      | 0.216| 0.387|      | 0.140| 0.473|      |
| SEM       | 0.020| 0.125|      | 0.045| 0.150|      | 0.062| 0.112|      | 0.040| 0.137|      |

a The samples were assayed in duplicate wells at the same time in three separate assays. All assays were valid, and the data for the pre- and postvaccination samples represent the mean values derived from the three independent assays. Fold, fold change; SEM, standard error of the mean for all readable pre- or postvaccination samples.
were not obtained for samples 15, 16, and 26, but in the two former samples the prevaccination titer was >0.1 IU/ml and in sample 26 the postvaccination titer was >0.1 IU/ml. In all five assays, a 4-fold increase in titer could not be measured for samples 20 and 28. However, for sample 20 all prevaccination levels were >0.1 IU/ml, and both pre- and postvaccination titers were <0.1 IU/ml for sample 28.

DISCUSSION

Although reports have suggested that the anti-diphtheria IgG antibody titer required for adequate protection is >0.1 IU/ml (27), several reports have suggested that sensitive quantitation of specific IgG levels between 0.01 to 0.1 IU/ml are also important. Antibody levels within this range provide minimal protection, and quantification may provide important clinical information.

There is debate as to whether in vitro techniques for measuring anti-diphtheria IgG antibodies are as sensitive as the in vivo neutralization tests. It has been suggested that inaccuracies with ELISA may be due to the detection of nonneutralizing antibodies directed toward an immunogenic but nontoxic part of the toxoid (22, 23). In these studies, it is not stated whether these ELISAs were calibrated to any standard, such as NIBSC 00/496. When using standardized in vitro ELISA for the measurement of anti-diphtheria IgG antibodies, the correlation of sensitivities with an in vivo neutralization assay and in vitro tissue culture method is good at values as low as 0.01 IU/ml (17, 30). At titers <0.01 IU/ml, however, the correlation has been shown to be poor (14, 17).

In the present study, we have compared the assay performance of five different commercially available anti-diphtheria toxoid IgG ELISAs. The data suggest that considerable variation exists between the five assays. The ranges of percent CV for both intraassays and interassays were 3.4% to 17.4% and 4.6% to 27.3%, respectively, with samples of antibody titers >0.1 IU/ml and 4.2% to 15.2% for samples <0.1 IU/ml. The serum sample data readings suggest that the Euroimmun and BS assays give similar results, but the results are significantly different from the Scimedx, Serion, and Virotech results. The sensitivities between the five assays were comparable, but the Scimedx assay had lower specificity with respect to the other four assays.

Comparison of the accuracy of calibration to the international reference standard NIBSC 00/496 revealed interesting results. Only three of the assays possessed <15% mean variation between the assay calibrator material and reference standard at anti-diphtheria IgG antibody levels >0.1 IU/ml. The other two assays showed a large mean variation of 25.4% to 44.3%. At levels <0.1 IU/ml, one assay had a mean variation of 19% and the other failed to detect the low-level anti-diphtheria IgG antibodies.

Analysis of the anti-diphtheria IgG levels in pre- and postvaccination serum further highlighted variation. Depending on the assay chosen, a sample could be interpreted as possessing a protective or nonprotective level of anti-diphtheria IgG antibodies, and an accurate assessment of fold increases in titers could be difficult.

A recent independent study has also highlighted the differences that exist between commercial anti-diphtheria IgG ELISA kits. Di Giovine et al. (6) assessed the performance of five commercial anti-diphtheria IgG ELISAs using samples with values determined in the gold-standard VCA. The correlation coefficients between the individual assays and the VCA ranged from 0.6 to 0.85, and in three of the assays the majority of positive VCA samples were <0.1 IU/ml. The data clearly demonstrated that manufacturer-dependent differences between anti-diphtheria IgG ELISAs exist.

Since all assays were performed by the same technician and the protocols for all commercial assays were similar, such variations that affect antibody-antigen interaction may arise at several points in assay manufacture. The variation in precision data suggests possible differences in antigen source, preparation, or plate coating techniques, and the differences in the data sets imply that different types of blocking agents may have been utilized or that plate blocking may have occurred to different extents. Some of the variation is undoubtedly due to differences in the setting of the calibration, and it is not disclosed by all manufacturers how the calibration has been set in regard to the use of an international standard. Potentially, the types of buffer systems used in the assays could also influence the antibody-antigen interaction. Almost certainly, the data suggest that there is a need for some level of agreed standardization in the preparation of these assays.

The sensitive measurements of anti-diphtheria IgG antibodies are important clinically for two reasons: assessment of immune status and measurement of immune response. The WHO has suggested that the level of anti-diphtheria toxoid IgG antibodies required for optimal protection from diphtheria infection is >0.1 IU/ml. However, the levels of circulating anti-diphtheria IgG antibodies required for basic immunity in humans has been estimated to be 0.01 IU/ml by the Schick skin test (12, 24, 25), and many studies have reported that levels in the general population are <0.1 IU/ml and in some cases <0.01 IU/ml (1, 3, 11, 19, 20, 21, 26, 28, 32). Maple and colleagues showed that from the age of 14 years up to 54 years of age, the percentage of the population (n = 3,088) with anti-diphtheria toxoid IgG levels >0.1 IU/ml decreased from ~40% to ~5%. Within the same groups, the percentage of the population with levels <0.01 IU/ml increased from ~10% to ~40% (15). A study by Edmunds and colleagues found similar results (7). In the present study, the standard deviation of the mean titer in 56 random serum samples and in the prevaccination samples suggested that a considerable percentage of samples have anti-diphtheria IgG antibody titers <0.1 IU/ml (between 5.3% and 66%, depending on the assay used).

The response of the immune system to the challenge of a vaccine can aid immunologists in their diagnosis of an immuno-deficiency (5). Studies have suggested that responders, with an efficient immune response, produce a 4- to 31.1-fold increase in specific antibody titer to diphtheria vaccine (2, 16, 26), whereas nonresponders, possessing a defective immune response, only show an approximate 2.6-fold increase in titer (16) or an antibody titer postvaccination of <0.1 IU/ml (27). Furthermore, two studies have suggested that the percentage of nonresponders to the diphtheria vaccine could be between 4% and 14% depending on the age group and vaccination regime (11, 20).

In conclusion, we have provided a study to evaluate the performance by five different commercial ELISAs in the mea-
measurement of anti-diphtheria toxoid IgG levels. Given the clinical use of the information derived from the test, it is essential that sensitive and accurate ELISAs are utilized. We conclude that among the commercially available anti-diphtheria IgG ELISAs tested in this study, there are significant differences that exist in regard to precision, relative sensitivity, relative specificity, and calibration. Further studies are required to understand the clinical implications that arise from the use of different ELISAs with differing levels of sensitivity and these significant manufacture-dependent differences.

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