Technical report

Time resolved fluorometric immunoassay, using europium labelled antihuman IgG, for the detection of human tetanus antitoxin in serum

P A C Maple, C S Jones, N J Andrews

Abstract

A time resolved fluorometric immunoassay (TRFIA) has been developed and compared with an in house enzyme linked immunosorbent assay (ELISA) and commercial ELISA (Bindazyme) for the detection of tetanus antitoxin in human sera. A panel of 132 sera submitted for routine testing was used. Scatterplots showed a high degree of correlation between all three assays, although some divergence of results was apparent for low titre sera when comparing in house ELISA results with Bindazyme ELISA and TRFIA results. The TRFIA appeared to be more sensitive than the in house ELISA, and the Bindazyme assay compared well with the TRFIA. The intra-assay precision of all three assays, in terms of percentage coefficient of variation (%CV), was between 2.0% and 4.0%. The interassay precision ranged from 5% to 8% for the in house ELISA, 13% to 19% for the Bindazyme assay, and 11% to 13% for TRFIA. Both Bindazyme and TRFIA assays were simple to perform, accurate, reproducible, and amenable to automation. A particular benefit of the TRFIA was its large dynamic range, enabling tetanus antitoxin values of 0.01 IU/ml to 50 IU/ml to be measured with just one dilution of serum. TRFIA appears to be a useful serological technique worthy of further development.

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Fluorometric immunoassays are used infrequently in microbiology laboratories in the UK, although they have found wide application in other areas of pathology, where the sensitive and specific detection of antibodies is important (personal communication, 2001, Perkin Elmer Wallac, Cambridge, UK). In theoretical terms, a fluorometric detection system is capable of achieving sensitivities at least an order of magnitude below colorimetric assays using the same enzymes, because fluorescent compounds can repeatedly produce a signal in a short space of time. Background fluorescence

by some of the components in serum (such as proteins and bilirubin) has been a major limitation preventing the widespread use of conventional fluorescence based immunoassays; however, the use of lanthanide chelates (for example, europium) and time resolved detection systems eliminates background interference, because europium decays over a much longer time period than natural fluorescence, and time resolution enables specific europium related fluorescence to be measured. A previous study,¹ in which a time resolved fluorometric immunoassay (TRFIA) was developed and evaluated for the detection of tetanus antitoxin in human sera, has described this methodology to be simple to perform, accurate, reproducible, and to be amenable to automation. Our laboratory tests a large number of sera for routine and serosurveillance purposes and, although the in house enzyme linked immunosorbent assay (ELISA) used at present is satisfactory, we wished to determine whether the claims made for TRFIA were justified. Using the Wallac DELFIA (dissociation enhanced lanthanide fluorescence immunoassay) system, a time resolved fluorometric immunoassay (TRFIA) for tetanus antitoxin was developed and its performance was compared with in house and commercial ELISAs.

Methods

TIME RESOLVED FLUOROMETRIC IMMUNOASSAY (TRFIA)

DELFLIA microtitration plates (catalogue no. 1244-550; A/S Nunc, Roskilde, Denmark) were coated with 100 µl/well purified tetanus toxoid (batch 99/1; National Institute for Biological Standards and Control (NIBSC), South Mimms, UK) at a concentration of 0.5 Lf/ml in carbonate buffer (pH 9.6). The coated plates were stored at 4°C overnight and then washed four times with DELFIA wash solution (catalogue no. 1244-114; Wallac Oy, Turku, Finland) using a DELFIA 1296-026 (Wallac Oy) platewasher. Diluted serum samples (1/100 and 1/1000 in DELFIA assay buffer; catalogue no. 1244-111; Wallac Oy) were loaded (100 µl) on to the plate, together with a range of dilutions (0.19 mIU/ml to

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25 mIU/ml) of first international standard antitetanus immunoglobulin, human (batch 26/488; NIBSC) and negative and positive control sera. An uncoated microtitre plate was also loaded to act as a control for non-specific activity. The plates were then incubated at room temperature for two hours on a mini orbital shaker (Stuart Scientific, Stone, UK) set at 125 rpm, washed four times, and 100 µl of europium labelled antihuman IgG conjugate (catalogue no. 1244-330; Wallac Oy), diluted in assay buffer, was added to all wells. After a further two hours of shaker incubation at room temperature, the plates were washed four times and 100 µl of DELFIA enhancement solution (catalogue no. 1244-105; Wallac Oy) was added to all wells. The plates were then shaker incubated for five minutes at room temperature and read using a 1234 DELFIA research fluorometer (Wallac Oy) and the counts processed by multicalc software, version 2.5 (Wallac Oy).

IN HOUSE ELISA

Nunc maxisorp immunoplates (Life Technologies, Paisley, UK) were coated with 100 µl/well purified tetanus toxoid (batch 99/1; NIBSC) at a concentration of 0.5 Lf/ml in carbonate buffer (pH 9.6). Tetanus toxoid was not added to the final column of wells, which were left uncoated to act as a control for non-specific activity; 100 µl/well of 1% dried skimmed milk (Marvel, Premier Brands, UK) in carbonate buffer was added to these wells. The coated plates were stored at 4°C overnight and then washed three times with 0.05% Tween 20 phosphate buffered saline (PBST) using a Labsystems Wellwash 4 MK 2 plate washer (Life Sciences International, Basingstoke, UK). All wells were blocked by the addition of 100 µl 1.0% dried skimmed milk in PBST and incubated for one hour at 37°C inside a humid chamber. The blocked plates were then washed three times with PBST and loaded with serum samples, first international standard antitetanus immunoglobulin, human (batch 26/488; NIBSC), and negative and positive control sera. Serum specimens and negative and positive controls were initially diluted 1/50 in sample buffer (0.1% dried skimmed milk in PBST) and serial twofold dilutions (final volume, 100 µl) were performed in the plate to a final dilution factor of 1/1600. The standard tetanus antitoxin was initially diluted 1/400 in sample buffer and serial twofold dilutions (final volume, 100 µl) were performed in the plate to a final dilution factor of 1/12 800. Only 1/50 dilutions of serum samples were added to the no antigen control wells. The loaded plates were then incubated for two hours at 37°C in a humid chamber and washed three times. Rabbit antihuman horseradish peroxidase conjugate (A8792; Sigma, Poole, Dorset, UK), diluted 1/2000 in sample buffer (100 µl), was added to all wells, and the plates were incubated for one hour at 37°C in a humid chamber. The plates were washed three times with PBST and then three times with PBS and 100 µl freshly prepared substrate (ABTS; A-9941; Sigma) was added to all wells. After 20

minutes in a dark chamber at room temperature, the plates were read at 450/620 nm using an Anthos 2001 plate reader and the data processed using Biolise software.

BINDAZYME ANTITETANUS TOXOID ELISA

Bindazyme antitetanus toxoid IgG kits (catalogue no. MK010) were donated for this study by The Binding Site Ltd, Birmingham, UK. The manufacturer's instructions were followed in performing these assays. Briefly, the kits were brought to room temperature and 1/100 dilutions of serum samples were prepared in sample buffer and loaded on to a transfer plate. The assay plates were loaded with kit calibrators, positive control serum, and test sera in duplicate, taking care to minimise the time interval between adding the first and the last sera. The plates were then incubated for 30 minutes at room temperature and washed three times using a Labsystem's Wellwash 4 Mk2 plate washer (Life Sciences International). Conjugate was added and after a further 30 minutes of incubation at room temperature the plates were washed and the substrate was added. The reaction was stopped by the addition of 3M phosphoric acid after 10 minutes of incubation at room temperature, and the optical density of each well was measured at 450 nm using an Anthos 2001 plate reader. A calibration curve was plotted and the antitoxin concentrations of the test sera and control were interpolated. The antibody concentration determined for the control needed to fall within the manufacturer's stated range for the assay to be valid.

STATISTICAL METHODS

Assays were compared on a log scale using Pearson's correlation coefficient with 95% confidence intervals (CI) and difference versus means plots with fitted regression lines. Inter-assay and intra-assay variation was measured using the coefficient of variation. The sample size of 132 was sufficient to enable an accurate estimate of the correlation between the tests (95% CI within $\pm 3\%$) and also to detect overall differences in the mean IU/ml between the assays of 12% with 80% power at a 5% significance level.

Results

A panel of 132 human sera submitted to our laboratory for tetanus antitoxin testing was assembled to evaluate two alternative test methodologies to the in house ELISA method that is currently used. Using the commercial Bindazyme ELISA, 130 evaluable results were obtained and with the TRFIA, 131 evaluable results were obtained. The antitoxin values obtained by the different assays were all in the form "international units/ml" (IU/ml) and to enable comparison they were $\log_{10}$ transformed and plotted against one another. Figure 1 shows three pairwise plots with the line of equivalence and the correlation coefficient calculated with 95% bootstrap confidence intervals. The scatterplots showed a high degree of correlation and agreement between all three assays, although some divergence of results was

Figure 1 Scatterplots with line of equivalence and correlation coefficient (R) with 95% confidence intervals (in parenthesis). (A) Comparison of Bindazyme enzyme linked immunoassay (ELISA) and the in house ELISA; (B) comparison of time resolved fluorometric immunoassay (TRFIA) and the in house ELISA; (C) comparison of Bindazyme ELISA and TRFIA.

Figure 2 Ratio versus geometric mean plots with regression line. (A) In house versus Bindazyme enzyme linked immunoassay (ELISA); (B) in house versus time resolved fluorometric immunoassay (TRFIA); (C) TRFIA versus Bindazyme ELISA.

apparent for low titre sera when comparing in house ELISA results with Bindazyme and TRFIA results. This was investigated further for the three comparisons by plotting the ratio for the paired results against the geometric mean and fitting a regression line, as shown in fig 2. The regression analysis showed that TRFIA gave results that were systematically slightly higher than the Bindazyme ELISA (average 12% higher; $p = 0.001$). The in house ELISA gave similar results to TRFIA for high titre sera (> 1 IU/ml); however, for low titre sera the results were lower ($\sim 23\%$ lower; $p = 0.006$, when the mean is 0.1 IU/ml). When comparing the in house ELISA with Bindazyme the results were similar in the middle range; however, for low titre sera (< 0.1 IU/ml) the results were slightly lower (12% at 0.1 IU/ml; $p = 0.09$), and for high titre sera the results were slightly higher (21% at 5 IU/ml; $p = 0.04$).

The intra-assay precision was measured for all three assays using the same two sera, which had antitoxin values of 0.09 IU/ml and 0.94 IU/ml as determined by the in house ELISA, and for all three assays the percentage coefficient of variation (%CV) was between 2.0% and 4.0%. Interassay precision was also measured for all three assays using internal quality control sera (IQC) specific to each assay. For the in house ELISA, the %CV of the IQC sera ranged from 5% to 8%, for the Bindazyme ELISA the %CV was in the range 13–19%, and for the TRFIA the %CV was

11–13%. The normal reference ranges of the assays, when used as specified in the methods section, were 0.04–10 IU/ml for the in house ELISA, 0.01–7 IU/ml for the Bindazyme ELISA, and 0.01–50 IU/ml for the TRFIA.

Discussion

The mouse neutralisation test (MNT) is considered the “gold standard” test for determination of tetanus antitoxin values²; however, in consideration of animal welfare guidelines³ such testing should only be carried out when absolutely necessary. ELISA has been shown to correlate well with MNT,⁴ and our in house ELISA is based on this methodology. For routine testing, we consider concentrations of tetanus antitoxin of 0.1 IU/ml or greater to be protective and tetanus antitoxin values less than 0.1 IU/ml to indicate susceptibility and the need for immunisation. Both Bindazyme ELISA and TRFIA could detect antitoxin values of 0.01 IU/ml, which is considered to be the lowest concentration of antitoxin to give protection against tetanus.⁵ Our 10 fold higher cut off point for tetanus protection compensates for the possible presence of non-neutralising antibody and takes into consideration reports of tetanus occurring in individuals possessing antitoxin concentrations in excess of 0.01 IU/ml.^{6,7} Non-neutralising antibodies may be directed against non-neutralising epitopes or be of low affinity and may influence the results of some assays⁶; however, Sesardic and Corbel⁸ have shown that

the neutralising epitopes in tetanus toxin are immunodominant and that little non-neutralising antibody is produced.

From our results, both Bindazyme and TRFIA assays performed in a similar manner and were more sensitive than our current in house ELISA. TRFIA requires a time resolved fluorometer, which is an expensive piece of equipment; however, using this technology we have developed TRFIA assays capable of detecting tetanus antitoxin in oral fluids. Furthermore, TRFIA assays can be developed that measure tetanus and diphtheria antitoxin concentrations at the same time.⁹ Regarding the Bindazyme ELISA, we have shown it to be a useful alternative assay to either of the assays developed by us, and this study provides an independent assessment of the test. Both Bindazyme and TRFIA assays were simple to perform, accurate, reproducible, and could be automated. Our assessment of TRFIA is that it is possible to increase the sensitivity beyond that of traditional indirect ELISA, it has none of the risks to health associated with radioimmunoassay, and it is amenable to screening large numbers of sera.

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