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Evaluation of INSTAND e.V.'s external proficiency testing program for tetanus and diphtheria antitoxin detection: Lessons for assessing levels of immunoprotection

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ABSTRACT

Objectives: The aim of this study was to evaluate the development and status quo of the quality of high throughput *in vitro* diagnostic testing for tetanus and diphtheria antitoxin antibody (ATX) concentrations based on external quality assessment (EQA) data.
Methods: We analyzed manufacturer-specific data of 22 EQA surveys—each for the detection of tetanus and diphtheria ATX—to check the diagnostic strength of the corresponding *in vitro* diagnostic systems.
Results: While the results were mostly well aligned, individual surveys showed widely dispersed ATX concentrations. The medians of manufacturer collectives deviated from the overall median by up to 8.9-fold in the case of diphtheria ATX and by up to 3.5-fold in the case of tetanus ATX. Such a distribution in the results is particularly critical in the cut-off range for immunity and may lead to an incorrect assessment of vaccination status.
Conclusion: These results were surprising as there are International Standards for both ATX; however, the results may be linked to the high ATX concentration of the reference material, which deviates considerably from clinically significant concentrations. To increase the accuracy and diagnostic strength of both assays, we recommend a recalibration of the test systems and verification of their traceability to the International Standards.

Introduction

Since their initial development, vaccines have proven to be an effective tool in combating infectious diseases. Of the various vaccines available, vaccines against tetanus and diphtheria toxins have become the most frequently administered since they were first generally distributed around the world in the 1940s (ECDC, 2009; Weinberger, 2016). Serological evaluation of the level of corresponding protective antitoxin antibodies (antitoxins; ATX) is used to determine the corresponding level of immune protection in vaccinated and non-vaccinated individuals (van Riet et al., 2008; Weinberger et al., 2013) and to assess the general effectiveness of vaccination formulas and immunization schedules (Weinberger et al., 2013; Anez et al., 2020). In addition, these vaccines can be used to evaluate immune function in patients with a suspected immunodeficiency (Kwon et al., 2012; Farmand et al., 2017). The *in vivo* neutralization test is the gold standard for assessing protective antibodies against tetanus in non-vaccinated animals as it measures the biological activity of ATX. Even though this test is the most sensitive method, no international standard protocol currently exists. It is also expensive and labor-intensive and requires a large number of laboratory animals (WHO, 2018). Diphtheria ATX can also be assessed by an *in vitro* neutralization test in cell culture (Miyamura et al., 1974), but this test is also time-consuming and requires cell culture facilities (WHO, 2009; Di Giovine et al., 2010).

As these two neutralization test methods are rather unsuitable for high-throughput measurements, other *in vitro* methods have been developed for rapid semiquantitative detection of tetanus and diphtheria ATX. These methods offer advantages in terms of cost, speed, usability, and adaptability to automated processes (Di Giovine et al., 2010). A good correlation between the different test

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systems and the neutralization assays has been reported for tetanus (Simonsen et al., 1986; Gupta and Siber, 1994) and diphtheria (Knight et al., 1986; Melville-Smith and Balfour, 1988). However, the *in vitro* methods are accompanied by a loss of sensitivity in low-level ATX concentrations compared to the neutralization assays; hence, the WHO recommends higher cut-off values when defining positive test results (WHO, 2009, 2018).

Sensitivity is not the only important quality criterion; harmonization and metrological traceability are equally crucial aspects of *in vitro* diagnostic measurements. Although International Standard preparations do exist for both ATXs (NIBSC code 10/262, 98/552, TE-3) (NIBSC, 2014, 2019a,b), test systems have been shown in recent years to have varying levels of sensitivity and accuracy (van Hoeven et al., 2008; Perry et al., 2009; Di Giovine et al., 2010). These publications highlight the need for external as well as internal quality-control programs as an important way to monitor the quality of laboratory results when assessing vaccination status and levels of immunoprotection. High-quality laboratory testing not only ensures a better monitoring of vaccination success but can also contribute to a reduction in costs (Epner et al., 2013).

The Society for Promoting Quality Assurance in Medical Laboratories e.V. (INSTAND) has managed external quality assessment (EQA schemes, also known as proficiency tests (PT)), for diphtheria and tetanus serology for several decades. INSTAND is one of three organizations in Germany that is designated as a reference institute by the German Medical Association.

Thus far, only the results of one EQA scheme for diphtheria ATX estimation have been published (Di Giovine et al., 2010), whilst comparable results for tetanus ATX detection are still lacking. In this study, we analyzed the manufacturer-dependent accuracy and reproducibility of diphtheria and tetanus ATX titer assessments based on aggregated data collected as part of the 22 independent EQA schemes conducted twice a year from 2008 to 2018.

Results

We analyzed the data from 22 EQA surveys for both diphtheria as well as tetanus ATX titers conducted between 2008 and 2018. Over time, the number of participants for tetanus ATX surveys remained constant at roughly 140 participants. They also remained constant for diphtheria ATX surveys at roughly 120. The general pass rates for both EQA schemes ranged from 63 % to 97 % with no clear tendency (data not shown). As 80 % of our participants used test systems from one of the five major manufacturers r0030, r0062, r0175, r0176, or r0239, we focused on these collectives when evaluating the timelines (Figures 1 and 2) and analyzing the coefficients of variation (CV) (Figure 3). To highlight low-titer samples, which are of major diagnostic relevance, the corresponding manufacturer-specific distributions are magnified (Figures 1c and 2 c only sample 1). Manufacturer r0239 first appeared in the 2011-T3 EQA survey but has since been used by an increasing number of participants and was thus included in this evaluation.

Analysis of manufacturer-based distribution of tetanus ATX levels

The semi-quantitative distribution of tetanus ATX levels for the five analyzed manufacturers was predominantly well-aligned in the EQA surveys conducted between 2008 and 2018 (Figure 1). In contrast to all other manufacturers, manufacturer r0030 tended to have medians in several surveys that were up to 50 % higher than the overall median. For sample 1 in survey 2015-T3, there was even an upward deviation of over 200 % (Figure 1c). In contrast to manufacturer r0030, manufacturer r0239 often delivered slightly lower medians compared to all other manufacturers. For the remaining three manufacturers, r0062, r0175, and r0176, the percentage of deviation for the tetanus ATX titer results was mostly low compared to the total median, with only a few exceptions. For example, there was a strong value distribution in survey 2010-T6 (sample 1) where the medians of all manufacturers differed from the total median by 10 % (r0175) to 67 % (r0030) (Figure 1a).

Focusing on the low-titer samples, a distribution of the manufacturer-based collectives around the diagnostic threshold could be observed in some EQAs. Consistent with the observed trends, manufacturer r0030 showed an upward deviation and manufacturer r0239 showed a downward deviation compared to the total median for sample 1 in EQAs 2018-T3 and 2018-T6 (Figure 1c). In contrast, the distinct deviations in tetanus ATX detection for manufacturer r0062 of - 30 % in sample 1 for the 2015-T3 survey and - 53 % in sample 2 for the 2013-T3 survey are more of an exception and should be regarded as minor compared to the major deviations described above.

Analysis of manufacturer-based distribution of diphtheria ATX levels

The manufacturer-dependent deviations in median results for diphtheria ATX detection were higher than for the tetanus serology. In addition, the individual manufacturers did not show a general tendency toward an over- or underestimation of titer concentrations in relation to the total median, but rather showed a fluctuation around the total median. This can be especially observed for manufacturers r0062 and r0030. The percentage of deviation to the total median fluctuated for manufacturer r0062 between − 80 % and 138 %. In more than one-fifth of the EQAs, the results for manufacturer r0030 had an upward or downward deviation greater than 100 %. The upward deviations exhibited especially extreme values of up to five times the total median of the collective (e.g., in survey 2015-T3 sample 2 (Figure 2c)). Such strong deviations were the result of a significantly higher detection of antibodies in low-titer samples. For sample 2 in survey 2015-T3, not only were the results for manufacturer r0030 strongly elevated, but collective r0062 also had a median titer of approximately 0.67 IU/mL, making it ten times higher than the total median.

Manufacturers r0175 and r0176 showed a good alignment in most EQAs with single exceptions, like manufacturer r0175 for sample 1 in survey 2016-T3 (Figure 2a) or manufacturer r0176 for sample 2 in survey 2015-T3 (Figure 2b). Manufacturer r0239, whose test system was first included in survey 2011-T3, tended to produce lower titer results in comparison to the total median.

Manufacturer-specific analysis of the coefficients of variation

To obtain an impression of the interlaboratory comparability, we calculated the CVs for each manufacturer collective. As we were unable to distinguish between possible errors occurring when the results were transcribed and 'real' measurement errors, we decided to exclude the upper as well as the lower 10 % of the results when analyzing the CVs. This prevented these results from distorting the values. When an EQA had fewer than ten participants, we did not exclude the marginal values. As CV development was comparable in samples 1 and 2, we focused on sample 1 for both ATXs (Figure 3).

For systemic reasons, extreme CVs are to be expected in the case of negative titer samples as the titer concentration is outside the calibrated range of the test systems. Therefore, the respective peaks in the CV timelines, like in survey 2010-T3 for tetanus and survey 2012-T3 for diphtheria ATX detection, do not need to be discussed with respect to diagnostic testing.

The CV analysis for manufacturers r0030, r0062, r0175, r0176, and r0239 between 2008 and 2018 showed less variation for tetanus ATX detection than for diphtheria ATX detection. In the

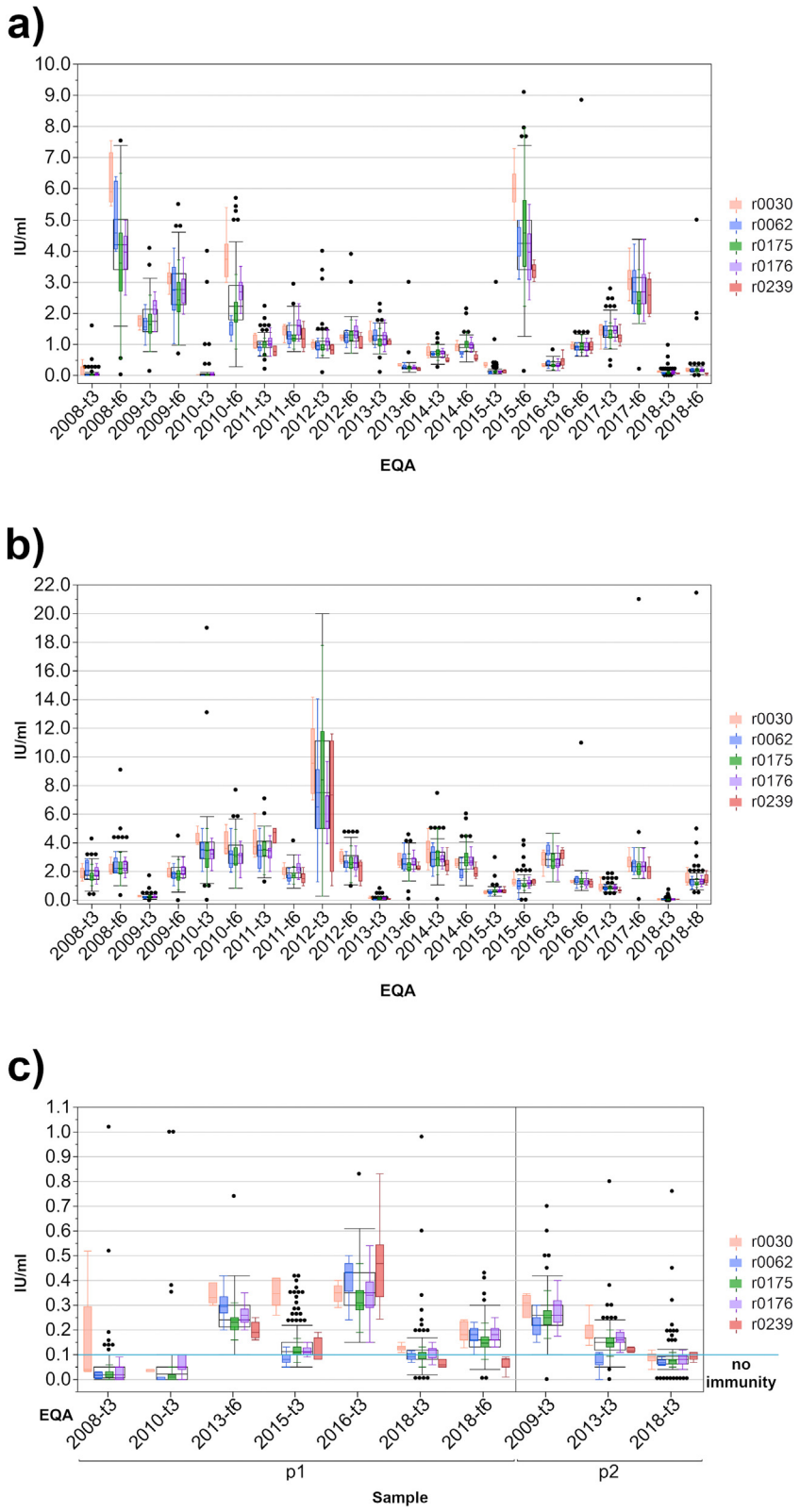

Figure 1. Analysis of the EQA results for tetanus ATX levels.
All EQA surveys between 2008 and 2018; (a) for sample 1 and (b) for sample 2, whereas (c) only shows examples of low-titer samples on a smaller scale. The black boxes display all results from the respective EQA schemes, and the distributions of specific method-based collectives are illustrated as smaller, colored box plots in overlay with the total results. Collectives are shown for the five main test systems r0030 (orange), r0062 (blue), r0175 (green), r0176 (violet), and r0239 (red). For all boxes, the whiskers stretch from the 1st quartile − 1.5*(interquartile range) to the 3rd quartile + 1.5*(interquartile range).

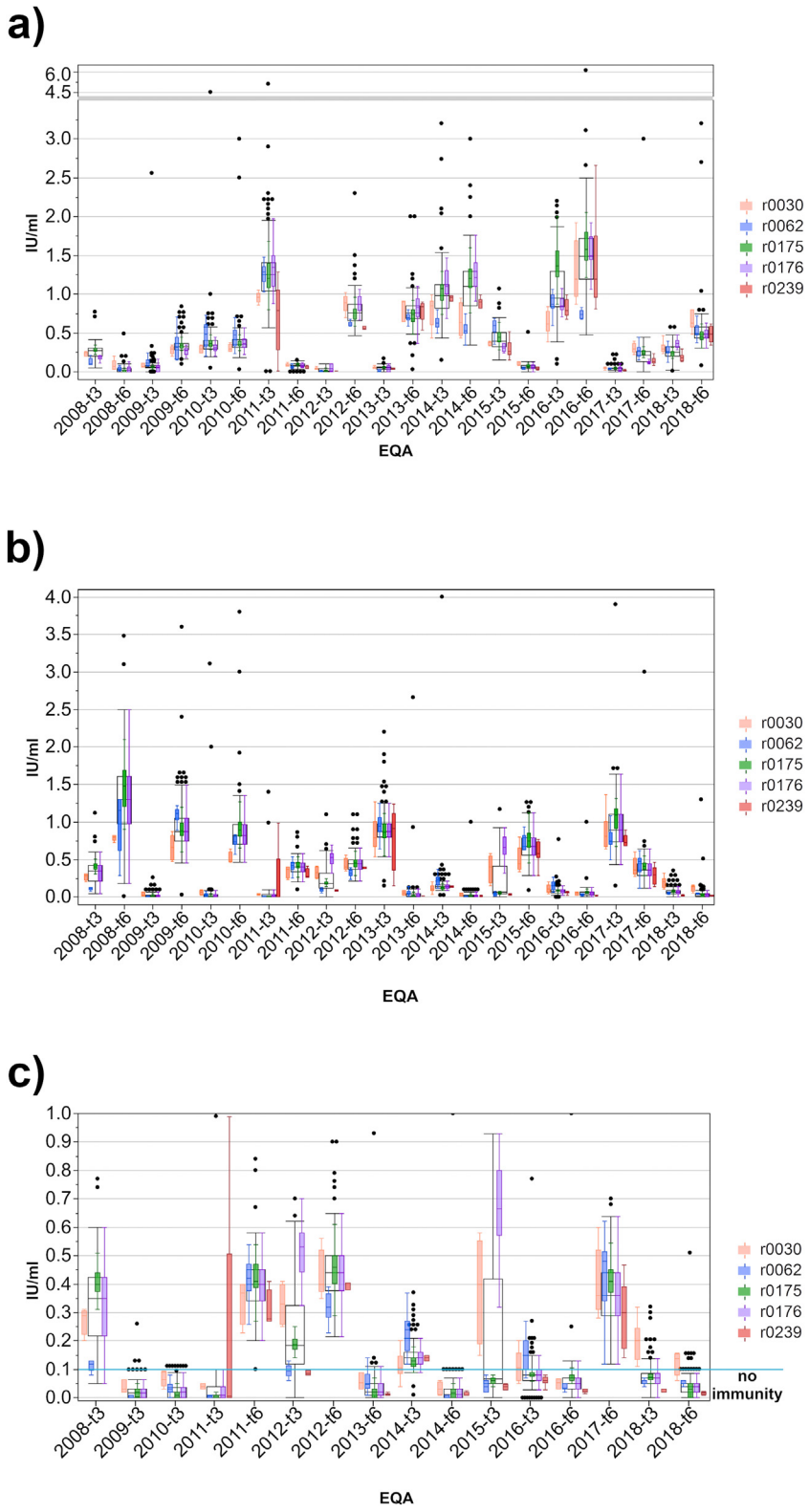

Figure 2. Analysis of the EQA results for diphtheria ATX levels.
All EQA surveys between 2008 and 2018; (a) for sample 1 and (b) for sample 2, whereas (c) shows examples of low-titer samples on a smaller scale. The big, black boxes display all results from the respective EQA schemes, and the distributions of specific method-based collectives are illustrated as smaller, colored box plots in overlay with the total results. Collectives are shown for the five main test systems r0030 (orange), r0062 (blue), r0175 (green), r0176 (violet), and r0239 (red). For all boxes, the whiskers stretch from the 1st quartile − 1.5*(interquartile range) to the 3rd quartile + 1.5*(interquartile range).

case of tetanus, CV values mostly did not exceed 20 %, except for EQA 2010-T3 (Figure 3a). Manufacturer r0239 showed the highest deviations of up to 40 %, but this is most likely due to the low number of participants. In contrast, the outlier-adjusted CVs for diphtheria ATX serology often reached values of 60 % to 85 % (Figure 3b). Here, manufacturers r0062 and r0176 had particularly

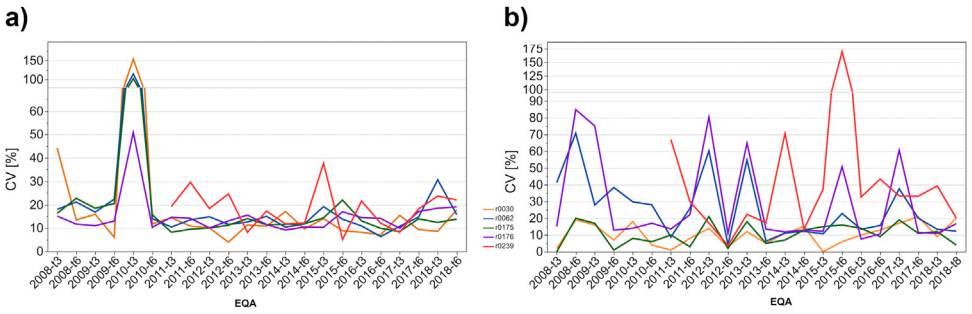

Figure 3. Interlaboratory comparison based on the coefficients of variation.
To determine (a) tetanus ATX and (b) diphtheria ATX for all EQA surveys conducted between 2008 and 2018 for the five main test systems r0030 (orange), r0062 (blue), r0175 (green), r0176 (violet), and r0239 (red).

striking numbers, with a strong fluctuation between low CVs of around 10 % and high CVs of 85 %. The CVs for manufacturer r0239 fluctuated in a similar manner, except for one prominent peak in survey 2015-T6. However, in subsequent years, the value remained below 30 % and thus even below the CVs for manufacturers r0062 and r0176. Manufacturers r0030 and r0175 had very consistent results, with CVs clearly under 20 %. This was comparable to their results of the tetanus testing.

Discussion

Diphtheria and tetanus are infectious and potentially fatal diseases, but preventable by vaccination against the symptom-causing toxins. The respective vaccines are among the most frequently administered vaccines worldwide and are well-understood in terms of their effectiveness and patient safety (ECDC, 2009; Weinberger, 2016). Examining ATX concentrations provides information on the patient's immune response and thus on the effectiveness of the immunization program (van Riet et al., 2008; Weinberger, 2016). At the same time, it serves as a way to assess immune response in individuals with a suspected immunodeficiency (Farmand et al., 2017; Blauvelt et al., 2019).

In this study, we analyzed the results of the INSTAND EQA schemes for tetanus and diphtheria ATX serology conducted between 2008 and 2018. We investigated interlaboratory comparability and manufacturer-specific accuracy and reliability. The general distribution of the results indicates relatively well-aligned manufacturer-specific results. Nevertheless, in individual EQAs, the manufacturer collectives showed distinct median deviations up to 3.5-fold for tetanus ATX and up to 8.9-fold for diphtheria ATX, which could adversely affect the clinical interpretation. The distribution of manufacturer collectives was often higher for diphtheria titers than for tetanus titers (Figures 1 and 2).

When determining vaccine status for tetanus and diphtheria, the WHO regards ATX concentrations $\geq$0.01 IU/mL, for which the neutralization tests are sensitive enough, as qualitatively positive. These concentrations thus provide a minimal protection against both toxins. In the case of other *in vitro* detection methods, a concentration of 0.1 IU/mL ATX is diagnostically relevant because this is the current decision point regarding whether immune protection is fully present. However, long-term protection requires an ATX concentration of $\geq$1.0 IU/mL; otherwise, a booster vaccination is recommended (WHO, 2014a,b, 2018).

Due to their clinical relevance, low-titer samples are regularly distributed in the EQA schemes to examine the performance quality of the participating medical laboratories around 0.1 IU/mL (Figures 1c and 2 c). Since 2008, strong deviations of several manufacturer collectives have been repeatedly observed for samples containing borderline ATX concentrations. As there have been no reports of any severe effects of a booster vaccination due to overvaccination, a false-negative result would most likely be harmless for the patient (WHO, 2018). False-positive results, on the other hand, could lead to individuals missing a booster vaccination and therefore to an unnoted loss of immune protection. Such a misdiagnosis is particularly unfortunate as inadequate vaccination coverage of the elderly is a known problem (Poethko-Muller and Schmitz, 2013; Weinberger et al., 2013; Filia et al., 2014). False-negative results for individual manufacturer collectives were observed in two tetanus EQAs and one diphtheria EQA. False-positive results for individual manufacturer collectives appeared more often for diphtheria than for tetanus serology. Since 2015, the false-positive results for diphtheria ATX have been cumulative in five of the seven negative titer samples. Manufacturer r0030 showed a higher probability of false-positive results for borderline sera of both ATXs than the other manufacturers. In some cases, the deviation in median values, in comparison to the collective with the lowest results, was up to 3-fold for tetanus ATX (sample 1, survey 2015-T3) and 4-fold for diphtheria ATX (sample 2, 2015-T3). Interestingly, in the diphtheria survey 2015-T3, all collectives were widely scattered, and manufacturer r0176 showed an even higher upward deviation than manufacturer r0030. Such dispersed results are rather the exception but indicate that the reproducibility of test batches might not be satisfactory and that test system calibration is insufficient. In the case of manufacturer r0030, this theory is further supported by the observation that, in contrast to the low-titer samples, the test system underestimated concentrations over 1.0 IU/mL for diphtheria ATX.

Differences in specificity or sensitivity of the ELISA tests might originate from the technique and the properties of the plate coating, the usage of different blocking agents, and the preparation and the purity of the utilized antibodies, as already described by Zasada et al. (2013). The aluminum-adsorbed toxin (WHO, 2017), which is used as the antigen in these test systems, is a key factor and might be the cause of the non-specificity (Miller et al., 2006). Therefore, suitability of the used antigen should be verified by the producers of the toxoid.

The manufacturer-based differences in both titer determinations examined in this study are comparable to previous findings. The observed inconsistency in manufacturer-dependent results for diphtheria serology can be confirmed by previous studies comparing the performance of ELISA tests with those of neutralization tests (Melville-Smith and Balfour, 1988; Skogen et al., 1999; Di Giovine et al., 2010). In the case of tetanus ATX determination, Perry et al. have described the potential for improvement in the comparability of the results from various ELISA test systems, as some tests tended to overestimate or underestimate (Perry et al., 2009). Scattered results in tetanus ATX titer determination, which can be accompanied by a misinterpretation of the immune status, were also found by van Hoeven et al. Additionally, they estimated the correlation of patient sample

results to those of reference preparations and observed consistently lower or higher results for individual ELISA tests (van Hoeven et al., 2008). Both publications use a dilution series of an international WHO standard for tetanus ATX as a reference in their assessment of testing accuracies. However, as these dilution series were obtained by adding distilled water, it cannot be ruled out that the results in these publications were influenced by matrix effects (Miller et al., 2018). In contrast, our proficiency tests contained unprocessed serum samples from individual donors whose vaccination status was known in most cases. Thus, matrix effects due to additives were unlikely.

Furthermore, our samples were thoroughly tested for homogeneity according to DIN EN ISO/IEC 17043:2010-05, and we observed no abnormalities in the corresponding samples.

Another purpose of protective antibody titer testing is to assess the effectiveness of vaccines and the corresponding vaccination programs based on the immune response of individuals. For pre-vaccination levels of 0.1 IU/mL or greater, an immune response to a tetanus or diphtheria booster vaccination normally achieves an ATX titer that is at least 4 times higher than the clinically relevant protective concentration of 0.1 IU/mL (WHO, 2014a,b). Furthermore, a maximum 4-fold increase in titer enables immunosuppressed individuals to be identified (Paris and Sorensen, 2007). Fortunately, with a few exceptions, the CVs we observed among manufacturers, were only up to 30 % for tetanus ATX and up to 80 % for diphtheria ATX (Figure 3). Thus, the current interlaboratory dispersion is far lower than the 4-fold titer increase, which indicates a normal humoral immune response. The assessment of the vaccine's general effectiveness as well as the humoral immune response should not be affected as long as initial and follow-up samples are measured in the same run of the same test system. Due to observed median differences of up to 8.9-fold for diphtheria ATX titers, we recommend always measuring pre- and post-immunization samples in the same run and with the same test to achieve comparable results.

In several surveys, manufacturer r0239 showed a larger scattering of titer results compared to the other four manufacturers in the study. This is not astonishing as manufacturer r0239 did not enter the market until 2011. However, in recent years, manufacturer r0239 achieved results for both titer detections that are on par with those of manufacturers r0062 and r0176. Since EQA 2016-T3, the CVs of manufacturer r0239 have been rather inconspicuous compared to the other manufacturers. Nevertheless, the observed scattering in the EQA results did not reflect any manufacturer's specifications, which purport inaccuracies up to a maximum of 12 % for positive or weakly positive sera, despite the presence of an international reference preparation for both ATX.

The existence of International Standards is most likely a key factor in the predominantly good harmonization of the manufacturer-specific results. The International Standard for Tetanus Immunoglobin (TE-3) has existed since 1992 (NIBSC, 2019), while the 1st International Standard for Diphtheria ATX (10/262) was not introduced until 2012 (Stickings et al., 2013). Thus, it is not surprising that the EQA results of the diphtheria ATX were more widely distributed than those of tetanus. However, implementation of International Standard 10/262 in 2012 did not lead to any improvement in measuring accuracy. Neither an improvement in the convergence of the different collectives (Figure 2) nor a permanent rise in inter-laboratory comparability (Figure 3) could be observed. Zasada et al. found that deviations in diphtheria titer determination, due to the use of different ELISA test kits, could be reduced by using International Standard 10/262 to calibrate the test system instead of the manufacturer's standard (Zasada et al., 2013). This indicates a deficit in the traceability of the manufacturer's calibration curves to the International Standard. Furthermore, as the standard material often contains artificial components for stabilization or spiking, insufficient commutability might potentially impact measurement accuracy and hence the calibration of the manufacturer's standards. International Standards TE-3 and 10/262 have supplemented the material of human origin with 5 % w/v freeze-–dried human IgG, which can induce matrix effects (Miller et al., 2006). Furthermore, as the current standard preparations are assigned concentrations of 120 IU/mL for tetanus ATX IgG (NIBSC, 2019) and 2 IU/mL for diphtheria ATX IgG (NIBSC, 2014), they need to be diluted to access the clinically relevant concentrations of around 0.1 IU/mL. This dilution process might further hamper the accuracy of the calibration due to matrix effects (Miller et al., 2018). Therefore, we strongly believe that the evaluation of a new international standard preparation with an ATX concentration in a clinically relevant range would further improve the clinical diagnostics of these two important ATXs.

Conclusion

Manufacturer-based alignment in the determination of tetanus and diphtheria titers is adequate, but in view of the availability of International Standards for both ATXs, there is room for further improvement. Despite the good overall alignment, stark discrepancies could occasionally be observed between the manufacturer collectives, which might have a negative impact on the assessment of an individual's current immune status. Manufacturer r0030 repeatedly displayed measurement inaccuracies, which is especially critical with regard to false-positive results for both tetanus and diphtheria ATX serology. Reproducibility needs to improve, especially with respect to determining diphtheria titers. The verification of the test systems, the suitability of the utilized toxoid, traceability to International Standards, and the development of International Standards with clinically relevant analyte concentrations might reveal further optimization potentials with regard to accuracy, reproducibility, and harmonization of tetanus and diphtheria ATX results.

Methods

External quality assessment procedures at INSTAND

The data from the EQAs were obtained from regular EQAs conducted worldwide. Each EQA participant received two samples with different analyte concentrations per EQA program. Participants reported on the qualitative and semi-quantitative results and could make a diagnostic comment on each sample (data on diagnostic comments were not analyzed).

The consensus value of all participants, calculated using algorithm A, was used to evaluate the semi-quantitative results of both EQAs. When a manufacturer-dependent variance was observed, collectives were formed and evaluated separately. EQA experts took into consideration an evaluation area of 40 % around the median for tetanus ATX and an evaluation area of 25 % for diphtheria ATX. With respect to the qualitative results, the participants had to indicate whether the samples were positive, borderline, or negative; the semi-quantitative results could be reported in ranges.

Sample material

Both positive and negative samples were obtained from voluntary blood donors. The samples tested negative for HIV, HBV, and HCV. No stabilizing additives were added (Müller et al., 2009). Homogeneity of each sample batch was tested according to DIN EN ISO/IEC 17043:2010-05 before the samples were used in the corresponding EQA (DIN, 2010). The patient's informed written

consent is available for the project. A positive vote from the ethics committee of Goethe University Frankfurt (Main) has been obtained for samples from voluntary blood donors.

Data evaluation and statistics

We evaluated 22 EQAs for tetanus ATX as well as diphtheria ATX, which were organized by INSTAND between 2008 and 2017. Both evaluations were carried out on a manufacturer-specific basis. The manufacturers were pseudonymized, and the codes are listed at https://www.instand-ev.de/no_cache/en/eqas-online/service-for-eqa-tests/. The groups can be filtered for EQA 310 (tetanus ATX) or EQA 318 (diphtheria ATX). An EQA survey and analyte must then be selected. The manufacturer codes can be found below the statistical data in the box marked “reagent” (“r”).

Values that exceeded the calibration curve by more than 20 % were excluded from the analysis because they were most likely transcription errors or methodical outliers. When analyzing the method and manufacturer collectives, we corrected obvious errors resulting from sample swaps so they would not distort the general quality of the test results. To evaluate the qualitative performance of the EQA participants, no corrections were made when analyzing whether the positive sample was correctly identified. For both analytes, a cut-off value of 0.01 IU/mL was used to define qualitative positive samples and a cut-off value of 0.1 IU/mL was used for protective immunity.

Basic statistical analyses were performed using jmp from SAS Institute (Cary, North Carolina, USA).

Generation of images

The overlay images were generated using Gnu image manipulator software 2.10.2.

Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Funding source

We did not receive any external funding sources for this project.

Ethical approval

For all serum samples, the patient’s informed written consent is available for the project. A positive vote from the ethics committee of Goethe University Frankfurt (Main) was obtained for samples from voluntary blood donors.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ijid.2020.12.046.

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