Meeting Report

WHO Working Group meeting on revision of the Manual of Laboratory Methods for testing DTP vaccines

World Health Organization, Geneva, Switzerland

20-21 July, 2006
1. Objectives and Scope

The meeting was opened by Dr D J Wood, Coordinator of Quality, Safety and Standards Team (QSS) of the Immunization, Vaccines and Biologicals Department (IVB). Prof R Dobbelaer was appointed Chairman and Dr M J Corbel as Rapporteur. Dr Wood stated that the objectives of the meeting were to review the methods currently available for determining the potency of diphtheria, tetanus and pertussis (wP) vaccines and to appoint a drafting group to revise the manual. He then described the current organization of the QSS team and its collaboration with other teams within IVB department as well as with other departments in the area of biological standardization. The DTP Manual needed to be seen in the context of developments in the immunization world. An estimated global coverage of 78% was reported for DTP immunization. Based on WHO/UNICEF estimates for 2004 coverage with full immunization for >90% of the population was achieved in 106 countries, with 80-89% coverage in a further 32 countries and 50-79% in 42 countries, with <50% coverage in 12 countries, mainly in Africa. There was room for improvement and introduction of new vaccines for a wider range of diseases could prevent many more deaths. The Global Immunisation Vision and Strategy (GIVS) initiative from WHO and UNICEF was designed to achieve this over the next 10 years. This required an integrated strategy involving the IVR unit, QSS and the EPI team. Within QSS there are three groups namely Norms and Standards for Biologicals (NSB), Vaccines Quality and Regulation (VQR) and Global Safety Concerns (GSC). NSB is responsible for development of new and replacement standards established through the Experts Committee on Biological Standardization (ECBS) and the promotion and implementation of WHO standards. VQR is in charge of strengthening regulatory systems for vaccines and development of regulatory pathways for priority vaccines. It is also responsible for pre-qualification of vaccines and immunization equipment. GSC is concerned with safety issues on a global scale and with improving the monitoring and management of adverse events. The biological standardization of vaccines was a major responsibility including global measurement standards, written standards and support for the evidence base for standards. The promotion and implementation priorities included the development of tools to assess the impact of WHO quality standards, producing/updating manuals of test procedures for vaccine quality and use of reference materials, making recommendations on stability of reference materials and aligning priorities of collaborating centres. The Expert Committee on Biological Standardization was due to meet in 2006 and would consider a proposal for the 4th International Standard for Whole Cell Pertussis Vaccine. Regional Standards were also under development in line with the WHO policy of de-centralization. The science base for norms and standards for DTP had been supported by meetings on DT potency assay (2002), standardization and control of pertussis vaccines (2003), updating of recommendations for D,T, P and combined vaccines (2003) and recommendations for whole cell pertussis vaccines (2005).

Dr D Lei (QSS/IVB/FCH, WHO) gave an overview of the current WHO manual for DTwP vaccine testing. This had been developed to provide guidance on procedures for laboratories with limited access to technology and had been widely used. However, it was recognized that various developments had occurred during the period between its inception and now. The objectives of the present meeting were to review the current
potency and safety test methods available for D,T and wP vaccines, to review sections of
the Manual dealing with laboratory tests for DTwP vaccines and for statistical analysis of
results and to establish a drafting group to update the Manual. He summarized the current
contents of the Manual and indicated factors to be taken into account in its revision. The
principles to be observed in selecting methods included identifying those critical for
assuring safety, efficacy and quality and which were consistent with WHO
Recommendations for production, quality control and evaluation of vaccines. Methods
that were well recognized but not yet included in current Recommendations should be
taken into account. These would include in vivo and/or in vitro methods for determining
potency, safety testing and identity. The current Manual comprises four parts: I). General
section, II). Potency control of viral vaccines III). Potency control of bacterial vaccines,
IV). Calculations and statistical analysis of results. The current objective was to revise
sections III and IV. It was necessary also to consider whether these sections should be
published separately from that on viral vaccines. After discussion, it was decided that
this would be the best course.

2. Review of current approaches in potency testing of DTP vaccines and
statistical consideration

Dr M J Corbel (NIBSC,UK) reviewed the procedures available for quality control testing
of DTwP vaccines. The purpose of QC procedures was to ensure that specifications for
quality, safety and efficacy were met. The manufacturers performed their own in process
and release QC and the National Control Laboratories (NCLs) performed confirmatory
testing. It was obviously desirable that effective and compatible procedures should
be used by both parties. The basic methods for testing DTwP vaccines were well-
established and over the years had been shown capable of ensuring the production of safe
and effective vaccines. They did, however, possess some disadvantages particularly in
relation to cost, animal availability and severity and attempts had been made to develop
alternatives. Some of the latter were in quite widespread use but the extent of their
standardization and validation was not always clear.

Review of the QC requirements for D and T toxoids identified the key issues as antigen
content/identity, freedom from toxicity, absence of reversion to toxicity and potency.
Various methods were available for monitoring these qualities and these were
summarized. A similar review was made for wP vaccines which identified the key issues
as assuring minimal toxicity, adequate potency and full representation of serotype
antigens. The methods currently available for monitoring these properties were also
identified. It was important to determine which methods were in common use and which
had fallen out of favour. Dr Lei had conducted a survey of control laboratories
worldwide to identify the current pattern of usage of tests and statistical methods and had
received information from 12 laboratories in Europe, Asia and North America. The data
obtained had enabled an overall impression to be formed. For D toxoids, flocculation
and immunodiffusion were the most popular methods for antigen content/identity
determination, with ELISA or other methods used by a few. For specific toxicity, guinea
pig assays were generally preferred, with rabbits or Vero cells used by a few. For
potency, serological response in mice was most widely used, with other laboratories
using lethal or non-lethal challenge or serological response in guinea-pigs. Programmed parallel line assay was by far the most popular method for statistical analysis followed by programmed probit analysis. For T toxoid antigen content/identity determination, an almost identical pattern was observed. For specific toxicity, guinea pig tests were the most widely used, with a small minority of laboratories using unspecified alternatives. For potency, lethal or non-lethal challenge or serological response in mice were preferred with a small number using guinea-pig challenge or serological response. Again, parallel line or probit analysis were the preferred statistical methods. For both D and T potency assays multiple dilution methods were preferred to single dilution assay.

For determination of wP serotype antigens/identity, agglutination was the preferred method, with others used infrequently. For safety testing, the mouse weight gain test was almost universally used. However, about half the laboratories used the histamine sensitization test and one third used lymphocytosis measurement. Most performed the LAL assay for endotoxin. Other safety tests were used infrequently. Only the active mouse protection test with multiple dilutions was used for potency determination. All laboratories used the programmed probit method for analysis except for two that used the Combistat package.

It was noted that no laboratories reported use of the passive haemagglutination assay and this was recommended for deletion from the Manual. In conclusion, it was suggested that the Manual should recommend the most widely used primary tests for identity, safety and potency. Alternative methods could be included where validated. Proven statistical methods should be recommended. Updated guidance should also be provided on the use of reference preparations and a standardized approach to protocol review.

Dr T Jivapaisarnpong (Ministry of Public Health, Thailand) reported on the approaches to potency testing of DTwP vaccines used by NCLs in developing countries. Most NCLs performed potency assays during the registration process, for lot release of locally produced products and sometimes for post-marketing surveillance. For lot release, the assay was used to monitor consistency of production and qualitative assays were preferred. For pertussis vaccine potency, the intracerebral challenge (Kendrick) method was used but high variability between tests was a problem. Validation of the method was necessary and the procedure, parameters and other acceptance criteria needed to be included in the Manual. For example recommendations are made that healthy mice should be used but this is not defined. It is recommended that an inbred strain should be used but none is specified. In practice, Swiss albino, ICR and ddy were widely used. Guidance on numbers of mice per group, weight, age and number of vaccine dilutions to be used, were more specific. Randomization of the mice was recommended and some NCLs used a computer programme for this but others did not randomize. No specific guidance was given on the choice of working reference materials and this was needed. It was stated that not more than 6% of mice should die before challenge but it was not clear if this meant 6% in each group or overall. In practice there were few deaths at this stage. It was recommended that each LD$_{50}$ dose should contain not $>$300 viable organisms but no minimum was specified. Mice dying within 72 h of challenge were to be excluded from the calculation. The number of deaths at this stage could be due to many factors
and an acceptable maximum should be specified. The pass criteria for the test define the minimum potency and lower fiducial limit but do not indicate a retest policy in the event of an unsatisfactory result. In the case of diphtheria and tetanus vaccines WHO recommended the multiple dilution method for registration but permitted single dilution assay for lot release once consistency was shown for 10 lots. In practice, most NCLs used the multiple dilution method throughout and few used the US NIH method. Clarification was needed on the validation procedure, the working reference preparation and the interpretation of potency in IU if the test was performed in mice. For the guinea pig assay, the minimum acceptable number per group needed to be defined and it was not clear how consistency could be established using a qualitative single dilution assay. A standard retest policy was required. In conclusion, developing country NCLs needed clear guidance on test procedures, method validation and data analysis, one standard method for each type of test, a quantitative assay using the minimum number of animals, the necessary computer software for statistical analysis and animal randomization and guidance on working reference preparations. Training and a proficiency testing scheme were also necessary to achieve consistent inter-laboratory performance.

Dr M-E Behr-Gross (EDQM, Strasbourg) summarized the EDQM policy on DTwP testing. The aim was to move towards a simplified lot release assay driven by application of the 3Rs in accordance with EC Council directives and conventions on animal experimentation. The consequences would be revision of Ph Eur monographs if supported by experimental data. The pre-requisites were validation of new methods and the availability of corresponding reference reagents. In 1996, projects were begun on establishing reference materials and alternative methods for D and T toxoid potency testing. The validation of serological methods for D and T vaccines was completed in 2004 and 2002 respectively. The guinea-pig model was chosen as offering many advantages over the mouse model. The method also was potentially applicable to combination vaccines as well as monovalent preparations, with considerable saving of animals, and it was intended to develop this aspect in the near future although there might be problems with some formulations. Further studies were needed to determine if the model could be used for wP vaccines. The EDQM was supporting an EVCAM validation study on a serological method for wP potency.

Prof R Dobbelaer (SIPH, Belgium) reviewed the procedures used by the Belgian NCL for assaying potency of D,T, wP and aP vaccines. The vaccines covered included monovalent, divalent D and T vaccines and combinations containing these with wP or aP and permutations of HepB, IPV and Hib. These were tested according to the European Pharmacopoeia. For D and T potency, the multiple dilution lethal challenge assay in guinea-pigs (D) and mice (T) was used. Calculation was by probit parallel line analysis in conjunction with validity criteria. In some cases, single dilution procedures were used to monitor consistency, in comparison with an in-house reference representing the minimum specification. The multiple dilution methods gave reasonable precision but wider variation was evident with the single dilution procedures.

wP potency was determined by the multiple dilution intra-cerebral challenge assay against a freeze-dried in-house reference calibrated in IU. Calculation was by the probit
parallel line model with validity criteria. The NCL had consistently achieved a higher degree of precision than the manufacturers but average potency results were in fairly close agreement. For aP vaccines a single dilution relative ratio immunogenicity assay was performed in mice against an in-house DTaP reference. Sera were assayed by ELISA and the pass criterion was a relative ratio not less than a historically established threshold. Hitherto, all batches had met this requirement.

Dr R Gaines-Das (NIBSC, UK) discussed the statistical considerations of potency testing. She noted that biological assays are comparative, requiring the estimation of a relative potency using a biological test system. It is essential that the variation inherent in such systems is quantitatively determined if estimates are to be correctly expressed. Thus statistical methods are essential. The existing Manual implicitly recognizes the need for statistical input into assay design and into quality control, assay validation, and standardization but does not explicitly deal with these issues. The application of the 3Rs can only be successful if approaches to it incorporate sound statistical design and validation. Dr Gaines-Das thus proposed that Part I of the Manual should be revised to explicitly recognize the need for statistical advice and expertise in the areas of assay design, method validation and quality control. Part I should also clarify that the Manual provides guidance and examples of good practice, but that there are other methods which could be used provided that they are appropriately validated. Part IV of the existing Manual is narrowly focused on calculation of assay results using classical statistical methods. These methods are essentially unchanged and continue to be appropriate for many assays. However, the changes in statistical software availability need to be recognized, and more emphasis on interpretation of results might be helpful for the user. Dr Gaines-Das therefore proposed that this section should be renamed Statistical Analysis of Results and begin with an introduction recognizing the broad role of statistics in assay design, validation, quality control and interpretation as well as in the calculation of results. The availability of various statistical software packages should be described. Areas which might be given more attention include design of assays with the need for appropriate randomization, alternate designs and analyses for assays carried out using 96-well microtitre plates, combination of estimates of potency and quality control of assays. It was further suggested, and supported by A Daas (Statistician, EDQM, written comments provided), that the utility of the Manual would be enhanced if worked examples, with output from various statistical software packages could be included. This would also provide data which could be used for validation of in house methods, and could be especially helpful to laboratories newly introducing particular assay methods.

3. Review current approaches in identity and toxicity testing of DTP

Dr D Sesardic (NIBSC, UK) reviewed the current approaches to determining identity and content of D and T antigens in DTP vaccines. Section A5 of WHO TRS 800 (1990) stipulates that the identity test must be performed on at least one labeled container from each final lot of vaccine. The primary purpose of the test at this stage is therefore to confirm presence of the correct antigens in the final stage of the production process. Any version of the immunoassay which is dependent on interaction between the antigen and specific anti-toxin may serve as an identity test and the most widely used methods
include flocculation and immunoprecipitation assays, such as SRD, but dot blotting and ELISA can also be suitably adapted. As most vaccines are adsorbed to mineral carriers, adsorbed antigen must be desorbed by a suitable method prior to use in an identity test. The method of choice at NIBSC is a highly robust SRD method with the limit of detection of approx 4-5 Lf/ml. This works well for most applications to confirm presence of antigens in final lots of vaccine but cannot specifically detect antigenic toxoid or provide the sensitivity to detect low levels of non-adsorbed antigens in vaccines. Improved assays with well defined functional monoclonal antibodies have been developed with the aim of ensuring consistency of product by providing more information on interaction between antigens and adjuvant in the final product. This was in line with recommendations made for DTP and combined vaccines (Amendments 2003, WHO TRS 927, 2005) to support an approach towards simplified potency models and to provide more information on antigen quality and degree of adsorption. Such an approach to quality control would be particularly attractive to NCLs and easily adopted by laboratories with limited access to technology.

Data were presented to demonstrate the advantages of the methodology which is considerably more sensitive than SRD, with a detection limit of close to 0.002 Lf/ml. Limitations were also considered in view of the fact that information is dependent on the amount of antigen desorbed and therefore is affected by the age of the vaccine and will not necessarily correlate with potency. It was stressed that antigenic toxoid is not the only component contributing to the protective immune response.

Examples were provided to demonstrate the successful application of technology to detect and quantify D and T antigens adsorbed to alum gel, after desorption with sodium citrate or EDTA, and when present in non-adsorbed form. Product specific profiles of adsorbed and non adsorbed antigen were determined for a wide range of vaccines and have established that antigen content correlates with antibody response during stability testing. Monitoring of D and T toxoids in several DTwP combinations identified similarities in profile and degree of adsorption for both antigens and confirmed that particularly for T, high antigen content did not always lead to the most potent product. Additional examples for other combined vaccines, excluding wP, have confirmed the value of the information with respect to amount of antigen present, antigen interaction with other components, and contribution to potency. Finally, studies on adult formulation Td vaccines have suggested an association of high antigen level and degree of adsorption with adverse reactions in use.

Dr D Xing (NIBSC, UK) reviewed the identity and in vitro toxicity tests available for the pertussis component of DTwP vaccines. Currently, apart from mouse weight gain test other tests such as Chinese hamster ovary cell (CHO-cell) test, histamine sensitization test and leukocytosis promotion test are also used for monitoring pertussis toxin (PT) toxicity in whole cell pertussis vaccine. The Limulus amoebocyte lysate assay (LAL test) and rabbit pyrogen test can be used for monitoring endotoxin content in whole cell pertussis vaccine. Data were presented to demonstrate the methodology of the CHO-cell assay. This assay has been used as an in vitro test for determination of residual active PT in vaccines, based on the morphological changes to CHO cells in the presence of active
PT. In this test, the CHO cells are treated with test vaccine dilutions and a range of dilutions of a PT reference. A reference vaccine is included as positive control and cells with no treatment serve as the negative control. After incubation, the degree of clustering of the cells is observed and scored under an inverted microscope. The highest dilution of the test vaccine showing total cell clustering represents the titre. The amount of active PT in the test sample can then be semi-quantified against the reference preparation of known concentration. Examples of assay validity criteria and interpretation of assay results were presented. The CHO-assay has the advantage that it is an in vitro assay, and shows good sensitivity and good reproducibility for standardized assays. However, it requires tissue culture facilities. High dilution is usually needed (at least 1/10) for whole cell vaccine because the presence of adjuvant in the vaccine may affect cell viability in the assay. Because of aggregation, the test may also fail to detect residual toxin present in toxoid after chemical detoxification.

The identity tests used for whole cell pertussis vaccine include agglutination, immunogenicity and immunodiffusion assays. However, the agglutination assay may not be suitable for testing final formulation because the presence of adjuvant in the vaccine may affect the end point observation. The immunogenicity test involves animal usage. An example of the in vitro immunodiffusion assay for identity of wP in final container vaccine was presented. In this test, as most vaccines are adsorbed to aluminum adjuvant, B. pertussis cells must be desorbed by treatment with tri-sodium citrate to remove the adjuvant and then the cell pellet treated with detergent to release the antigens into the supernatant prior to use in an identity assay. Based on the interaction between the pertussis antigens and specific anti-pertussis serum, an immunoprecipitation line will form in an agarose immunodiffusion plate and therefore confirm the presence of pertussis antigens. The detection limit for the assay is \(~0.08\) single human dose (SHD).

Dr D Sesardic (NIBSC, UK) described in vitro and in vivo assays for assessment of toxicity of diphtheria vaccines. According to the WHO TRS 800 (1990), safety tests for diphtheria vaccine are required and performed for control of bulk purified toxoid (as detailed in sections A 3.4.3 and A3.4.4) the purpose of which is to ensure absence of active toxin and that reversion to toxicity cannot take place on storage at elevated temperature. Specific toxicity is also required for the control of final bulk (as detailed is section A 3.5.5) the purpose of which is to ensure freedom from toxin and reversion in the final product. Guinea pigs provide a suitable model for both applications and are essential for testing of final product. Alternatively, a cell culture assays may be used for control testing of bulk purified toxoid, provided that sensitivity of the test is demonstrated to be not less than that of the guinea pig test. Dr Sesardic pointed out that only with the use of a suitably calibrated reference diphtheria toxin can cell culture assay provide assurance of sensitivity and reproducibility. Such an approach has been adopted by the Ph Eur from 2005. In addition to diphtheria toxin specific toxicity, studies at NIBSC have confirmed that diphtheria toxoid, together with Hib conjugate, are the main components of DTaP combinations contributing to induction of IL-6 and TNF-\(\alpha\) from human monocytes. The response is highly donor specific and the significance of this finding is still unclear.
Dr Sesardic pointed out that an alternative assay for tetanus toxin is still at the early stages of development. The strategy adopted at NIBSC focused on characterization of enzymic activity of toxin L chain and in identification of differentiated cell lines of neuronal origin, in view of still limited knowledge on the nature of the protein high affinity receptor. More development work is required, however, before suitable *in vitro* models of toxicity could be recommended for validation.

4. **Strategy for WHO Recommendations on Combine vaccines**

The final session considered the strategy to be followed for the WHO Recommendations on Combined and Combination vaccines.

Dr E Griffiths (Health Canada, Canada) reviewed the current Recommendations on DTP combined vaccines. He distinguished between ‘combined’ vaccines such as DTwP and ‘combination’ vaccines that contained DTwP or DTaP plus components such as Hib, IPV or HepB. The current WHO requirements for DTP cover DTwP (WHO, 1990). The ECBS was currently in process of updating the whole document which was revised in 1964, 1979 and 1989. Amendments to the D and T sections had been adopted in 2004 and those to wP in 2005. The current Guidelines on aP vaccines were to be upgraded to Recommendations in updated form. The current DTP Recommendations dealt with combined vaccines but did not deal effectively with combinations. How this could be done needed consideration. There were problems in standardization and control of D and T potencies with no globally accepted approach. However, a practical approach is followed. The 1964 Requirements specified assay against an International Standard vaccine but with no minimum potency required although some national regulations specify not less than 30 IU for D vaccines. These Requirements were modified in 1979 to give a definite requirement of 30 IU for D and 40 IU for T vaccines with adjuvant. In 1990 confidence intervals were added and single dilution assays were permitted for routine release once a consistency track record was established. Validated *in vitro or in vivo* toxin neutralization tests were also permitted. Currently, the Ph Eur follows the WHO specification but the USA uses a serological assay requiring a minimum response but with no reference vaccine. Several WHO/EDQM consultations had tried to resolve the differences and evaluate progress with alternative methods. However, the positions were unchanged and some countries followed US and others WHO/Ph Eur recommendations. Suggested simplifications to lot release were under debate with technical issues to be decided. Amendments to the 2004 Recommendations included an updated section on International Reference Preparations and separation of D and T potency determination into licensing and batch release sections. For licensing, full potency assay in guinea pigs or use of a validated serological assay in mice was specified with potencies expressed as previously. For monitoring consistency post licensing, assays using reduced numbers of animals could be used subject to NCL/NRA agreement. Consistency data needed to be supported by physical/chemical methods and periodic review. Recommendations for wP were adopted in 1989, were subsequently reviewed and were being updated in the light of current knowledge. Agglutinogens were now identified as fimbriae 2 and 3 and reference reagents (polyclonal and monoclonal antibodies) were available. Better markers were available for phase 1 organisms. Although
electronic/optical methods were available, the Opacity Standard was still considered useful. The Kendrick potency assay was retained in the absence of a validated alternative but with encouragement to use humane end points. The specification was a minimum mean potency of 4 IU per SHD with a lower fiducial limit of 2 IU. For toxicity testing, the mouse weight gain test was emphasized, with improved details of methodology. Other tests were also possible. Monitoring of detoxification and consistency of production through pertussis toxin and endotoxin assay was encouraged. New sections on stability evaluation and non-clinical evaluation and clinical evaluation of new products were included. Revisions to the Manual needed to capture these changes. For combined DTP, tests take account of antigen interactions with a higher specification for T potency (60 IU). For aP based combinations the issues were still under consideration. Currently, specifications were available for all individual components (Hib, IPV, HepB) but it needed to be decided if an additional Manual was needed to deal with combinations containing them. Possibly additional Manuals would be needed for viral vaccines (M, MR, MMR, MMRV) and new conjugates (MenA, C, ACWY). Advice was sought on the strategy to be followed.

5. Discussion on development of WHO references for Diphtheria and Tetanus

Dr D Sesardic (NIBSC, UK) discussed the WHO reference standards for D and T toxoids and the need for separate or combined reference preparations. It was noted that the 3rd WHO IS for Diphtheria toxoid, adsorbed, established in 1999, is in limited supply and replacement has already been initiated at NIBSC by adding a new project to the standards data base. EDQM have also identified a need for replacement of the same standard in view of the fact that the current Ph Eur BRP is the same material as the current WHO IS and because of limited availability. Recent studies with 3rd WHO IS for Tetanus toxoid, adsorbed, established in 2000, have identified problems in use suspected to be due to the high moisture content. Both standards will therefore require replacement, although replacement of Diphtheria toxoid is considered as much more urgent. As most vaccines today contain at least Diphtheria and Tetanus components it was suggested that it may be possible to consider making one divalent standard, rather than two monovalent formulations. It was discussed and agreed that the primary purpose of any WHO replacement standard will be to act as a primary reagent for calibration of national, regional or product specific standards and as such it may be more useful for this to remain monovalent. EDQM have also expressed preference to have monovalent formulations. Dr Sesardic confirmed that the shift towards potency estimation by serology assays will be more reliant on product specific working standards but it will be essential to maintain specifications set by the WHO IS and therefore the need for stable standard formulations will remain and it will probably be easier to continue with monovalent formulations. It was agreed that trial formulations could be made containing monovalent and divalent forms but any progress towards making divalent WHO ISs will need to be supported by data and agreed by consensus.

The requirement for a diphtheria toxin standard for determination of absence and irreversibility of toxoid was also discussed by Dr Sesardic. The diphtheria toxin standard is essential for an in vitro cell culture assay of toxicity, to monitor sensitivity and reduce
inter- and intra-assay variability. Suitable toxin of high purity and stability has been formulated at NIBSC and adopted in 2003 as the first Ph Eur Biological Reference Reagent based on in vivo and in vitro data provided by 11 laboratories in 7 countries. Currently there is no WHO standard for this purpose, although the cell culture method is mentioned in WHO TRS 800 (1990). The WHO 1st IRR for diphtheria toxin, known as STT, established in 1955, is still available in limited quantities but never established as a reference standard for in vitro assays of toxicity. In view of the suitability of the Ph Eur reagent, it was agreed that the same material could be considered for adoption as a WHO IRR and be given a value based on ED50 in Vero cells. Dr Knezevic requested information on the extent to which such material would be used by non-European laboratories. In view of the fact that the cell culture assay is only suitable for testing of purified diphtheria toxoid and most non-European manufactures still rely on the guinea pig model it was likely that a reference toxin would not be used by many laboratories, at this stage. However, it is clear that establishment of a standard would help towards wider adoption of in vitro alternative methods. Dr Sesardic noted that well defined diphtheria toxin is also required for other applications, such as potency challenge in vivo and toxin neutralisation assays for anti-toxins but most laboratories use in house preparations and calibration of such materials in defined units is not essential for their use.

6. Review current approaches in identity and toxicity testing of DTP

This was followed by a detailed discussion of revision of the Manual. It was agreed that the DTwP testing sections should be separated from the sections on viral vaccine testing that would be more appropriate in a separate Manual. A plan for revision and updating of the various sections of the DTwP Manual was devised and small specialist groups set up to deal with the Introduction, DT Testing, wP testing, statistical methods and validation issues, respectively. Dr Kreeftenberg (Holland) noted that the mouse based assays for toxoid potency were still quite widely used and it was desirable to establish appropriate standards for these to enable the results to be related to the standard guinea pig assays. Dr S Gairola (SII, India) pointed out that not all of the methods in the current manual were used by manufacturers.

During a general discussion of the current Manual, the control of potency of bacterial vaccines, traditional methods for assaying potency of adsorbed D and T toxoids and pertussis vaccines in monovalent or combined form, serological assays for D and T toxoids and the Vero cell method for potency assay of D toxoid were considered at length. It was noted that monovalent wP vaccines were no longer available and did not need to be included in the Manual.

The outline of the revised Manual was proposed:

Outline of the revised Manual of control testing for DTP vaccines

Title:

Contents:
Methods which are in the WHO Recommendations/Requirements, and otherwise validated procedures. The Manual will cover tests for potency, safety/toxicity and identity/antigen content of DTwP vaccines with the following chapters.

Outline of each methods session:
1. Introductory part, including: purpose of the method, principles,
2. Materials (reagents, critical reagents, buffers, animals and references),
3. Procedures,
4. Calculation (specify the statistical method), (parameters to be monitored)
5. Validity of the test,
6. Retest, (refer to specifications)
7. Combining results
8. Validation specific for this method and acceptance criteria (parameters), suitability for the given product
9. References

I. General Introduction:
1. Scope
2. Status of the document in relation to guidelines
3. Validation of assays and technicians
4. Standardization of testing and importance of references in bioassay, and calibration of in-house references
5. Use of laboratory animals (reference for monitoring the microbial infection)
6. Lab safety
7. Quality assurance and control of testing laboratory
8. Statistical considerations (general)
9. Validation of mouse assays for the potency testing of D and T vaccines

II. Testing of diphtheria vaccines:
1. Potency
1.1 Challenge (lethal and intra-dermal) testing in guinea pigs
1.2. Serological assays in guinea pigs
1.2.1 Immunization of animals
1.2.2. Titration of immune sera by Vero cell assay or ELISA
1.2.3 In vivo toxin neutralization method
1.3. Serological assays in mice
2. Toxicity testing
2.1 In vivo specific toxicity and toxicity reversion in guinea pigs or rabbits
2.2: Vero cell testing for absence of toxin and reversion
3. Identity test and Lf
3.1. Flocculation test (Ramon and laser light scattering)
3.2. Radial immunodiffusion
3.3. Rocket immunoelectrophoresis
4. Antigen content
4.1 ELISA

III. Testing of tetanus vaccines
1 Potency
1.1 Challenge (lethal and paralysis) testing in guinea pigs
1.2. Serological assays in guinea pigs
1.2.1 Immunization of animals
1.2.2. In vivo toxin neutralization method
1.2.3. Titration of immune sera by ELISA
1.2.4. Titration of immune sera by ToBI
1.3. Serological assays in mice
2 Toxicity
2.1. In vivo specific toxicity and toxicity reversion in guinea pigs
3. Identity and Lf
3.1.1. Flocculation test (Ramon and laser light scattering)
3.1.2 Radial immunodiffusion
3.1.3. Rocket immunoelectrophoresis
4 Antigen content
4.1 ELISA

IV. Testing of pertussis vaccines
1 Potency
1.1 Kendrick test
2. Specific toxicity testing
2.1 Mouse weight gain testing
2.2 Other tests
2.2.1 CHO cell for PT
2.2.2 Histamine sensitization
2.2.3 Lymphocytosis promoting
2.2.4 Endotoxin LAL
3. Identity test
3.1 Agglutination
3.2 Immunodiffusion

V. Statistical analysis
Introduction, with emphasis on assay design and randomization
Probit assay for quantal responses
Parallel line assay
Single dilution assays (Statistical tests for comparison of two groups)
Combination of estimates (retest and repeat test)
Interpretation of statistical output
Quality monitoring of assays
Software
Additional topics for inclusion:
Validation of mouse assays for the potency testing of D and T vaccines
Calibration of secondary reference materials

Deadlines:
First draft will be submitted to WHO by 6th October 2006.
Statistics group will provide their input by 15 December.

Drafting group:
I. General introduction
   Dr R Dobbelaer, Dr M J Corbel
II. Testing of diphtheria vaccines
    Dr T Sesardic, Dr R Winsnes, and Dr H Kreeftenberg
III. Testing of tetanus vaccines
    Dr T Sesardic, Dr R Winsnes and Dr H Kreeftenberg
IV. Testing of pertussis vaccine
    Dr D Xing, Dr Y Horiuchi and Dr T Jivapaisarnpong
V. Statistical analysis
    Dr R Gaines-Das, Dr Y Horiuchi
Validation of mouse assays for the potency testing of D and T vaccines
    Dr Hans Kreeftenberg

Notes:
1. SOPs: WHO QSS will send an email to participants to request provision of in house SOPs to drafting group to assist the drafting.

2. In the context of the Manual use the same terminology as in WHO requirements, recommendations and guidelines.

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Bibliography


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