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Meeting Report

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

Geneva, Switzerland

20-21 July 2006 and 28-30 March 2007



First meeting

The meeting on 20-21 July 2006 was opened by Dr D. J. Wood, Coordinator of Quality, Safety and Standards (QSS) of the Immunization, Vaccines and Biologicals Department (IVB), WHO. Professor 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 group and its interaction with other sections of WHO, particularly those concerned with immunization, vaccines and biologicals.

The DTP Manual needed to be seen in the context of developments in the immunization world. An average global coverage of 78% was reported for DTP immunization. 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 Immunization 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 team 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 Expert Committee on Biological Standardization (ECBS) and the promotion and implementation of WHO standards. VQR would strengthen regulatory systems for vaccines and develop pathways for priority vaccines. It was also responsible for pre-qualification of vaccines and immunization equipment. GSC was 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 developing 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 decentralization. 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 since its inception. 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 with 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/Requirements. 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.

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 to be 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 in 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 was the preferred statistical method. For both D and T potency assays multiple dilution assay designs 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.

Mrs 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 variability between tests was perceived to be 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 LD₅₀ dose should contain not >300 viable organisms, but no minimum was specified. Mice dying within 72 hours 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 design for registration, but permitted a single dilution assay for lot release once consistency was shown for 10 lots.

In practice, most NCLs used the multiple dilution design 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 on 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 the revision of Ph Eur monographs if supported by experimental data. The pre-requisites were the 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.

Professor 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 designs 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 the 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.

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 absorbed to mineral carriers, absorbed antigens 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 the presence of antigens in final lots of vaccine but cannot specifically detect antigenic toxoids or provide the sensitivity to detect low levels of non-absorbed antigens in vaccines. Improved assays with well-defined functional monoclonal antibodies have been developed with the aim of ensuring the consistency of the product by providing more information on the 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 absorption. 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 the 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 absorbed to Al gel, after desorption with sodium citrate or EDTA, and when present in the non-absorbed form. Product specific profiles of the absorbed and non-absorbed antigens were determined for a wide range of vaccines and have established that the 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 absorption for both antigens and confirmed that particularly for T, a 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 the 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 absorption 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 the mouse weight gain test other tests such as the Chinese hamster ovary cell (CHO-cell) test, the 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 the endotoxin content in the 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 the 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 a 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 the whole cell vaccine because the presence of the 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 the whole cell pertussis vaccine include agglutination, immunogenicity and immunodiffusion assays. However, the agglutination assay may not be suitable for testing the final formulation because the presence of the 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 the identity of wP in the final container vaccine was presented. In this test, as most vaccines are absorbed with the 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 the assessment of toxicity of diphtheria vaccines. According to the WHO TRS 800 (1990), safety tests for the diphtheria vaccine are required and performed for the control of the bulk purified toxoid (as detailed in sections A 3.4.3 and A3.4.4), the purpose of which is to ensure the absence of the active toxin and that reversion to toxicity cannot take place on storage at an elevated temperature. Specific toxicity is also required for the control of the final bulk (as detailed in section A 3.5.5), the purpose of which is to ensure freedom from the toxin

and reversion in the final product. Guinea pigs provide a suitable model for both applications and are essential for testing of the final product. Alternatively, a cell culture assay may be used for control testing of the bulk purified toxoid, provided that the 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 a 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 the diphtheria toxoid, together with Hib conjugate, are the main components of DTaP combinations contributing to induction of IL-6 and TNF- α 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.

During a general discussion of the current Manual, the control of potency of bacterial vaccines, traditional methods for assaying potency of absorbed 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 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 an 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 adjuvants. 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 the 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 the 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 the 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 necessary to deal with combinations containing them. Possibly additional Manuals would be needed for viral vaccines (M, MR, MMR, MMRV) and new conjugates (Men A, C, ACWY). Advice was sought on the strategy to be followed.

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, absorbed, established in 1999, is in limited supply and replacement has already been initiated at NIBSC. 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, absorbed, established in 2000, have identified problems in its use, suspected to be due to the high moisture content. Both standards will therefore require replacement, although replacement of the 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 a 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. It will be, however, essential to maintain specifications set by the WHO IS. 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 was 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 the 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 neutralization 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.

This was followed by a detailed discussion on the 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 the 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 J. G. 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.

Recommendations for Action

The title, content and the outline of the revised manual was proposed as shown in the appendix (see appendix).

Second meeting

Following the first meeting in July 2006, separate drafting groups provided the first drafts based on the basic principles agreed on that occasion. The working group again met in Geneva on 28-30 March 2007 to review the drafts and to input further suggestions or amendments to the contributions of the drafting groups.

The second meeting was opened by Dr I. Knezevic (WHO, QSS). Dr J. G. Kreeftenberg (Netherlands Vaccine Institute, Holland) was appointed Chairman and Dr Y. Horiuchi (NIID, Japan) was appointed Rapporteur. Dr Knezevic summarized the background and role of WHO in providing and promoting the implementation of norms and standards, especially in the dynamic field of vaccine development. The complexity of current formulations of DTP vaccines, and the need for continuing regulatory research were emphasized. She overviewed current global developments in biological standardization, stressed the importance of medicinal products of good quality for public health and clarified the role and responsibility of WHO in biological standardization, which is essential for assessing quality of vaccines and biologicals.

Currently, written standards for various vaccines are under revision and will be completed by 2008. Regarding measurement standards, guidelines for preparing secondary reference materials are under development. Providing guidance documents including manuals, and facilitating development of secondary reference materials, would improve access to international standards. WHO is adopting a holistic strategy in its approach to biological standardization.

DTP vaccines are fundamental for paediatric immunization, either alone or for use in combination with various other vaccines. Special advice would be needed to address the QC of multi-combination vaccines. The WHO recommendations for DT and wP have been updated recently. At present, work on revising the aP guidelines is underway, aimed at updating recommendations for improving QC test methods, and also for performing clinical evaluation of new aP vaccines. The laboratory manual for testing DTP vaccines would complement the WHO recommendations to assist NCLs, NRAs and manufacturers in the quality assessment of DTP vaccines, and would also serve as an example for manuals for other vaccines. Dr Knezevic anticipated the revision of the DTP recommendations as the next step for which the first draft was expected to be available by December 2007 for circulation for comments, and to be finalized in 2008.

Dr D. Lei (WHO, QSS), who has responsibility for coordinating the revision programme for the manual, summarized the objectives of the meeting and the current position following the previous meeting in July 2006. The revision of manuals is in accordance with the revision of WHO recommendations. The manual was intended to comprise a

general introductory chapter followed by individual chapters for Diphtheria, Tetanus and Pertussis testing and Statistical analysis, and draft versions of these were available from the individual drafting groups for discussion by the working group, based on the proposed outline from the meeting in July 2006.

The draft section for the general introduction prepared by Dr R. Dobbelaer and Dr M. Corbel was presented by Dr. Corbel. The revised chapter had been reduced and several sections were replaced by references to more recent documents.

Initial discussions were wide-ranging as the scope and purpose of the manual were considered. Content of the revised introductory chapter was recommended as follows:

- Scope of the manual, to provide general guidance.
- WHO has recently been developing guidance on the preparation of secondary standards, and reference to this was more appropriate than inclusion of these details in the present manual.
- Some safety considerations could be covered by reference to other guidelines, but the need for vaccination of staff working with DTP vaccines was noted.
- Information on quality control can be found by reference to WHO guidance on GMP, QA, QC and the various ISO guidelines.
- The quality of animals used for vaccine testing, suitability of animals, animal facilities and related issues are generally covered by animal regulations and requirements and this manual was not considered the appropriate forum for detailed discussion of these issues. The working group agreed that throughout the manual the term 'healthy animals' should be used to describe animals required for the test. Further details should not be specified, but the range and types of animals used in practice should be indicated.
- Methods for cell culture and preparation of hyper-immune animal serum would be moved to the sections describing tests which used these.
- An additional section on statistics was provided for this chapter by the statistics drafting group.

The working group noted the need for a glossary to define terminology used in this manual, and the drafting group for the general introductory section would address this. Several items were discussed including the various meaning given to the term validation, and the need to clarify the meaning of terms used in the manual.

In the course of discussing the tests to be included in the manual, the working group agreed not to cover physical and chemical tests, but suggested that it may be appropriate for WHO to develop a general manual for these methods.

The issues raised by 'combination' vaccines, which now include multiple components in addition to D, T and P may require special consideration. At the time when the manual was initially prepared, the 'combination' referred to was of D, T and P. Combined vaccines may now include a number of other components, such as IPV, Hib, HepB.

Information on the possible interactions between these components and the DTP components on the test methods given in the manual is limited and would need to be dealt with as a separate issue.

The proposed chapters for Diphtheria, Tetanus, Pertussis and Statistics chapters were considered individually and discussed in some detail.

The draft section on pertussis vaccines prepared by Dr Xing, Mrs Jivapaisanpong and Dr Horiuchi was presented by Dr Xing on behalf of the drafting group. She emphasized that the purpose of this section is to provide general guidance and/or examples to laboratories on how to set up the tests, consistent with the scope of this manual. It would be impossible to include all individual laboratory procedures in detail in this manual and laboratories will adapt the method according to their own conditions.

The main changes made in the new revision draft were summarized. Appendices on preparation of frozen aliquots for challenge culture and calculation of bacterial concentration in the challenge suspension were included in the revision. New sections on specific toxicity tests and identity tests have been added to the manual. Following discussion, it was agreed that the methods for acellular pertussis vaccines should not be covered by this manual. The Kendrick test remains the 'gold standard' potency test for whole cell pertussis vaccine. The method described under the mouse weight gain test should follow the procedure stated in WHO TRS 800. Other tests for monitoring pertussis toxicity described under the section of specific toxicity tests, e.g. CHO cell, are not regulatory tests, and this will be clearly noted.

Dr Sesardic (NIBSC) presented the draft sections on tests for diphtheria and tetanus vaccines. She summarized the principle and structure of these sections. For the diphtheria section, assays for toxicity, identity and Lf and antigen content were included and new sections on Vero cell and Elisa (guinea pig potency) and the toxin neutralization test *in vivo* (guinea pig) methods were added. It was noted that in the approach used by the USP, potency is expressed in IU as defined by the International Standard for diphtheria anti-toxin, and there is no estimate of potency in terms of IU of the IS for diphtheria vaccine absorbed. She emphasized that the current WHO IS for the diphtheria and tetanus vaccines, absorbed have assigned activity calibrated in the guinea pig challenge tests. Mrs T. Jivapaisarnpong (MOH, Thailand) noted that guinea pigs may not be available for routine batch release test in developing countries. There was discussion on how the IU (vaccine activity) can be used in such cases. Dr Kreeftenberg pointed out that lot release can be performed using various validated assays including assays in mice (WHO TRS 800 and 927). The use of methods and animal species to determine potency if guinea pigs are not readily available was discussed at length. It was agreed by the working group that the guinea pig challenge assay remains the 'gold standard' assay. The use of alternative validated methods may lead to the need for 'product specific' references. These issues would be addressed in more detail with a separate transferability paragraph in the assay validation section of the statistics chapter.

Serological assays for the testing of diphtheria and tetanus vaccines were also described by Dr Sesardic. To avoid confusion, the antibody titre obtained from these assays should be expressed in suitable terms and not in terms of an anti-diphtheria or an anti-tetanus IU.

Dr Sesardic covered the principles of drafting the section of the manual for tetanus vaccines with reference to the current manual and to other relevant documents. It was agreed that the guinea pig challenge assay remains the 'standard' assay for the calibration of reference standards. There are considerable similarities between the methods for diphtheria and tetanus and similar issues arise when other methods or animal species are used to determine potency. Much discussion was focused on the transferability of the IU based on the guinea pig challenge model to the mouse model for diphtheria and tetanus potency estimation. The group recognized the need for clear guidance in the manual on these topics.

Dr Rose Gaines Das (NIBSC) and Dr Y. Horiuchi (NIID) had developed the proposals for the revised statistics chapter. Sections of the previous manual were retained as far as possible, with some changes in order and focus. Formulae were not popular with the majority of users but were necessary for providing information on the procedures on which the calculations in this manual were based. The importance of assay design and randomization for obtaining unbiased results that reflect population values was emphasized. In the proposed revision, sections on parallel line analysis of quantitative responses and probit analysis have been adapted from the previous version, with additional examples and discussion of interpretation of results. Previous separate sections on comparison of two groups in the context of the single dilution assay have been combined and this section has been generalized to consider reductions intermediate between the full assay and the single dilution assay.

New chapters have been developed on retesting and combination of estimates, on assay validation, including the issues of transferability of IU as discussed for D and T vaccines, and on quality control with consideration of consistency of calculated parameters. The previous version included a chapter on computing software. Due to the changing technology in this area, and the wide availability of a range of software packages and web based open source software, this topic is now covered in the introductory general chapter. Throughout the chapter there has been an emphasis on additional relevant examples, with discussion on the interpretation of the statistical results. The participants in the working group agreed to provide additional data and examples. Expansion of relevant sections to incorporate reference to the 3Rs (Reduction, Refinement and Replacement) was also agreed.

During the presentations and discussions the need for a number of amendments and clarifications was noted. The relevant drafting groups would execute these amendments and forward the new versions to Dr Lei for collation into the draft version to be circulated for external review. Target dates were set for the end of April 2007 for completion of the meeting report, and for the end of June 2007 for completed revision of the draft manual.

Appendix

Proposed outline of the Manual for quality control of diphtheria, tetanus, pertussis and combined vaccines

Title:

Manual for Quality Control of Diphtheria, Tetanus, Pertussis and combined vaccines.

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 section:

- 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)

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 and mice
 - 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

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- II. Testing of diphtheria and tetanus vaccines
(Dr. Dorothea Sesardic, Dr. Randi Winsnes and Dr. Hans Kreeftenberg)
- III. Testing of pertussis vaccine
(Dr. Dorothy Xing, Dr. Teeranart Jivapaisanpong and Dr. Yoshinobu Horiuchi)
- IV. Statistical analysis
(Dr. Rose Gaines Das and Dr. Yoshinobu Horiuchi)

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