Meeting Report

WHO Workshop on Laboratory Testing of Diphtheria, Tetanus & Pertussis (DTP) Combined Vaccines

Jakarta, Indonesia

28-30 May 2014
**Introduction**

The World Health Organization (WHO) Workshop on laboratory testing of diphtheria, tetanus & pertussis (DTP) Combined Vaccines was convened from 28 to 30 May 2014, in Jakarta, Indonesia.

Participants in the workshop included the representatives from National Regulatory Authorities (NRAs), National Control Laboratories (NCLs) of vaccine producing, procuring and United Nations supplying countries, and WHO. The workshop was opened by Dr Roy A. Sparringa (Head of the National Agency for Drug and Food Control, the Republic of Indonesia), who welcomed the delegates. The workshop was chaired by Mrs Teeranart Jivapaisarnpong (Institute of Biological Products, Department of Medical Sciences, Ministry of Public Health, Thailand).

Dr Dianliang Lei (WHO) presented an overview of the development and regulation of combined vaccines in terms of the broad use of DTP based combinations as part of the Expanded Programme of Immunisation (EPI). He also discussed the effect new formulations (such as the addition of Hep B, Hib, IPV etc) can have in terms of immune interference, stability (component vs. combination), product specific standards, quality control (QC) strategy, clinical evaluation and new QC methods. The workshop was convened following requests from WHO Member States for direction on the implementation of new WHO Recommendations which include new QC methods for potency assay and toxicity, information on consistency of production and using International Units (IU). There was also a requirement for a statistical analysis programme for potency assays. The objectives of the workshop were therefore, to update the NCLs with the principles in WHO recommendations for diphtheria, tetanus, pertussis and combined vaccines and the Manual for Quality Control of DTP Vaccines; to introduce a statistical analysis programme used in quality control of vaccines; and to identify any potential needs for further guidance on quality control or any other relevant regulatory activity. At the conclusion of the workshop it was hoped that the participants would have a better understanding of the key principles of WHO recommendations on combined vaccines and recommended laboratory testing methods for combined vaccines; and participants would identify the current gaps in their laboratory testing and statistical analysis methods for quality control of DTP and combined vaccines.

Dr Lei also discussed WHO activities on Biological Standardization in relation to supporting NRAs as they independently control the quality of vaccines in accordance with the six specified critical functions defined by WHO. These functions consist of:

i. marketing authorization and licensing of activities: Approval of production facilities and approval of medicines for marketing;

ii. lot release: approval of biological products on a lot to lot basis;

iii. access to laboratory testing as needed;

iv. regulatory inspections: compliance with Good Manufacturing Practices (GMP), Good Clinical Practices (GCP), Good Laboratory Practices (GLP) and Good Distribution Practices (GDP).
v. regulatory oversight of clinical trials: Authorization and monitoring of trials and evaluation of clinical data;
vi. post-marketing activities: Monitoring of safety and efficacy including surveillance of Adverse Events Following Immunization (AEFI).

Details of WHO written standards that have recently been published, or are in development, were also presented along with information on the network of WHO Collaborating Centres for Vaccine Standardization. Finally, recent organizational changes within the structure of the WHO were also addressed.

Mrs Jivapaisarnpong invited each of the participants in turn to give a brief summary of the DTP combinations currently used in their countries, along with the source of these vaccines, the number of local manufacturers and the laboratory capability of each participant. A summary of the responses can be seen in Table 1.

Table 1. Current status of DTP vaccine supply, manufacture and control

<table>
<thead>
<tr>
<th>Country</th>
<th>Vaccine production and supply</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bangladesh</td>
<td>Import WHO PQ DTwP vaccines for use in EPI programme and aP combined vaccine for use in private sector only and two local manufacturers that are ready to fill bulk produced elsewhere.</td>
<td>NCL established in 2012 and not yet functional for laboratory testing. Protocol review is performed for lot release purpose.</td>
</tr>
<tr>
<td>China</td>
<td>Five domestic manufacturers and import from one foreign manufacturer. Several manufacturers are developing aP and regulatory requirements currently being established.</td>
<td>Approximately 50 million doses imported/year; 7 million doses released/year (domestic production). Test approximately 150-200 batches/year. Identity, sterility on every batch. 10-20% batches selected for potency testing (T – mouse challenge test, D – mouse Vero cell assay, P – MICA and immunogenicity). Active programme of new assay development: e.g. PT activity by CHO cell assay (also for antitoxin neutralisation assay) and E-HPLC.</td>
</tr>
<tr>
<td>India</td>
<td>DTwP vaccines from multiple local manufacturers in addition to imported products.</td>
<td>Work carried out under ISO 17025. Perform potency, sterility and identity on every wP batch. No experience testing aP (work in progress).</td>
</tr>
<tr>
<td>Indonesia</td>
<td>One local manufacturer but also</td>
<td>Have been performing testing on</td>
</tr>
<tr>
<td>Country</td>
<td>Vaccine production and supply</td>
<td>Notes</td>
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<tr>
<td>Iran</td>
<td>One domestic manufacturer producing DTP for &gt; 40 years – but has temporarily halted P component production for upgrading the production line and currently all DTP is imported (WHO PQ from e.g. India and Indonesia).</td>
<td>NCL has been performing potency testing using challenge method since 2005 but chose to switch to NIH method for D and T because of animal supply and animal house issues; Mouse Vero cell assay and T ELISA assay are planned to be established in NCL. No methods established for P – testing of this component performed by domestic manufacturer lab. Significant problems procuring WHO IS (e.g. from NIBSC) – related to International Sanctions and WHO will work with relevant authorities to try and resolve this specific issue.</td>
</tr>
<tr>
<td>Republic of Korea</td>
<td>Five- Six domestic manufacturers for DTaP (and DTwP solely for export).</td>
<td>NCL performs laboratory tests and protocol review and has introduced concept of risk assessment for the lot release programme. Potency test is performed on up to 20 lots (to demonstrate consistency) and then an exemption is applied such that only the first batch of the year is subjected to a potency test.</td>
</tr>
<tr>
<td>Malaysia</td>
<td>DTP (including aP combinations) vaccine is imported from several manufacturers.</td>
<td>NCL performs Protocol review only – intend to establish potency tests for DTP in the future.</td>
</tr>
<tr>
<td>Myanmar</td>
<td>WHO PQ DTP vaccines are imported.</td>
<td>FDA performs QC testing on imported products.</td>
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<tr>
<td>Pakistan</td>
<td>Only T vaccine is produced domestically; WHO PQ DTP is imported.</td>
<td>No lot release testing performed on imported (PQ) products. Domestically produced T vaccine is subject to laboratory testing by NCL. Intend to establish DTP tests for post-market surveillance of imported DTP vaccines.</td>
</tr>
<tr>
<td>Philippines</td>
<td>All DTP vaccine is imported.</td>
<td>NCL has minimal laboratory facilities so imported batches are subject to protocol.</td>
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<tr>
<td>Country</td>
<td>Vaccine production and supply</td>
<td>Notes</td>
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<tr>
<td>Sri Lanka</td>
<td>All DTP vaccine is imported (WHO PQ); aP combinations used in private sector only.</td>
<td>Imported lots subject to protocol review and occasionally sterility and innocuity testing. Trend analysis of manufacturer potency data is also performed.</td>
</tr>
<tr>
<td>Thailand</td>
<td>No domestic manufacturer of DTP at present – all DTP is imported (WHO PQ); aP combinations are also imported for private use.</td>
<td>For imported vaccines only the appearance test is performed routinely in addition to protocol review; sterility testing is performed as part of PMS in the case of adverse events. Challenge (WHO) testing is performed for whole cell pertussis and tetanus vaccine component but NIH test is used for the diphtheria component because of problems with supply of guinea pigs. D and T potency. aP by ELISA.</td>
</tr>
<tr>
<td>Viet Nam</td>
<td>One domestic manufacturer produces DTwP, and WHO PQ vaccines are also imported.</td>
<td>Abnormal toxicity and chemical tests on local vaccine. Potency, toxicity and identity test is performed on one in every five lots; challenge test is used for tetanus and wP components and the mouse Vero cell assay is used for the diphtheria component. No methods established for aP potency – lack of standards highlighted as a barrier to setting up test.</td>
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</tbody>
</table>

**WHO recommendations for diphtheria, tetanus, pertussis (DTP) combined vaccines**

Dr Stickings, National Institute for Biological Standards and Control (NIBSC), United Kingdom, presented a brief review of the revised WHO recommendations to assure the quality, safety and efficacy of diphtheria vaccines, tetanus vaccines and DT-based combined vaccines. These revised documents were published in WHO Technical Report Series 980 (2014) and are publicly available via the WHO website (Table 2).

Table 2. Revised recommendations to assure the quality, safety and efficacy of diphtheria vaccines, tetanus vaccines and DT-based combined vaccines
<table>
<thead>
<tr>
<th>Document</th>
<th>Hyperlink</th>
<th>Source</th>
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<tbody>
<tr>
<td>Recommendations for tetanus vaccines</td>
<td><a href="http://www.who.int/biologicals/vaccines/Tetanus_Recommendations_TRS_980_Annex_5.pdf?ua=1">http://www.who.int/biologicals/vaccines/Tetanus_Recommendations_TRS_980_Annex_5.pdf?ua=1</a></td>
<td>WHO TRS 980 (2014)</td>
</tr>
</tbody>
</table>

The main changes that were introduced in the revised versions of these documents were highlighted and included:

- a change in terminology in the title from “Requirements” to “Recommendations”;
- an update of the section on manufacturing recommendations;
- inclusion of a new section on non-clinical evaluation;
- inclusion of a new section on clinical evaluation.

For D and T vaccines, the main change to the section on manufacturing concerned the recommendations for potency testing. For both D and T, the minimum requirement for potency stated in the revised documents applies to the lower limit of the 95% confidence interval. It was noted however, that this would not have any impact for currently licensed products since the recommendations are not retrospective. The section on potency testing also now includes a large print statement that product specific potency specifications are acceptable provided they are appropriately justified and subsequently approved by the relevant regulatory authority.

Another significant change that has been incorporated into the revised document for tetanus vaccines is the option to perform the potency test in mice without the need to demonstrate transferability from a guinea pig model. This reflects the fact that the current WHO IS for tetanus toxoid, adsorbed (08/218) has a (separate) potency value assigned for both guinea pig and mouse assays. This standard can therefore, be used directly in a mouse potency assay, or for calibration of a local standard using a mouse assay – provided the appropriate unit value is used and traceability to the IS is maintained. It was also pointed out that this was not the case for diphtheria potency testing where the recommendation to demonstrate transferability between guinea pigs and mice remains (where mice are used for potency testing). This issue was discussed in more detail during the session on laboratory testing of DT vaccines. This session was followed by a short discussion and the main questions/comments are summarised in Table 3.

Table 3. Questions/Discussion – WHO recommendations for diphtheria, tetanus, pertussis and combined vaccines
### Question/Comment

<table>
<thead>
<tr>
<th>A concern was raised regarding the sections on clinical evaluation which recommend the use of pre-clinical lots for clinical evaluation and that this would necessitate full GMP during non-clinical evaluation.</th>
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</thead>
<tbody>
<tr>
<td>The WHO recommendation presents an ideal scenario but it is accepted that this will not be possible in many cases. For that reason, the recommendations for D and T also state that “where this is not feasible, the lots used clinically should be comparable to those used in the non-clinical studies with respect to the manufacturing process, immunogenicity, potency, safety, stability and other relevant characteristics of quality.”</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Now that the 4th WHO IS for Tetanus Toxoid Adsorbed has been established, do local standards calibrated against the 3rd IS need to be re-calibrated?</th>
</tr>
</thead>
<tbody>
<tr>
<td>No – they should remain in use until local stocks are exhausted. Replacement batches should be calibrated against the current WHO IS at the time of calibration.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>The choice of an appropriate comparator vaccine in clinical studies was discussed – particularly the case where no licensed comparator exists for a new combination.</th>
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<tbody>
<tr>
<td>This is covered in the revised recommendations to assure the quality, safety and efficacy of DT-based combined vaccines. In some cases, more than one comparator vaccine – administered concomitantly may be required. For assessment of a new aP vaccine, the recommendations to assure the quality, safety and efficacy of acellular pertussis vaccine (TRS 979, Annex 4) should be consulted.</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>The assigned units for the 4th WHO IS for Tetanus Toxoid Adsorbed were discussed and it was noted that the potency for the 3rd IS in guinea pigs and mice was very similar but that the potency for the 4th IS in guinea pigs and mice was different (almost 2-fold).</th>
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<tr>
<td>WHO International Standards are calibrated against the previous standard. The 3rd IS for Tetanus Toxoid Adsorbed (98/552) was calibrated against the 2nd IS (TEXA-2). The 4th (08/218) WHO IS was calibrated against the 3rd IS (98/552). Calibration of each standard represents a new comparison and there is no reason to expect the relative potency to remain constant.</td>
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**Quality control methods for potency and toxicity testing of diphtheria and tetanus vaccines**

Dr Stickings presented an overview of potency and toxicity testing of diphtheria and tetanus vaccines, with a focus on methods described in the WHO Manual for Quality Control of Diphtheria, Tetanus and Pertussis Vaccines. Copies of the presentation slides were distributed to all participants. Potency testing for diphtheria and tetanus vaccine is performed using well established challenge or serological methods in guinea pigs or mice. A number of participants had experienced problems with supply of guinea pigs. This has caused...
significant problems for validation of a mouse assay for potency testing of diphtheria vaccine which should only be done after demonstration of transferability according to the current WHO Recommendations for diphtheria vaccine. The situation is less problematic for potency testing of tetanus vaccines where mice are more widely used and the decision to assign “mouse IU” to the WHO IS for Tetanus Toxoid Adsorbed means that a mouse model can be used without the need to demonstrate transferability (subject to use of an appropriately calibrated reference vaccine). The use of homologous (or product specific) reference preparations was discussed in some detail – and although this can offer some advantages in terms of assay validity and harmonization, it was acknowledged that their use must be carefully considered and that there may be associated logistical difficulties for NCLs who would potentially need to manage multiple reference preparations for different products they control. These and other issues were discussed in a round table discussion on laboratory testing of DT vaccines and the main questions/comments are summarised in Table 4.

Table 4. Summary of round table discussion – Laboratory testing of diphtheria and tetanus vaccines

<table>
<thead>
<tr>
<th>Question/Comment</th>
<th>Response</th>
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<tr>
<td>A number of questions were raised on the topic of diphtheria potency testing in mice and transferability between mice and guinea pigs. The WHO recommendation that transferability should be demonstrated where mice are used for the potency test (instead of guinea pigs) is difficult to follow in many countries where there are considerable difficulties obtaining a suitable supply of guinea pigs.</td>
<td>Currently, the WHO IS for Diphtheria Toxoid Adsorbed only has an assigned unitage based on calibration in guinea pigs – there are no units assigned for mice. Although the recommendation to demonstrate transferability is scientifically justified, it is acknowledged that this is difficult to achieve in many cases. WHO acknowledge that this is an issue that needs to be resolved, although that might only be possible when the current WHO IS for Diphtheria Toxoid Adsorbed is replaced. In the meantime, NIBSC will work with WHO to see whether the current WHO IS can be assigned units based on calibration in mice. In the short term a local NRA may approve use of the mouse Vero cell assay (in the absence of demonstration of transferability) if appropriately justified. However, it was also noted that potency testing in mice could be problematic – for example lack of parallel dose response and differences in potency estimates depending on the mouse strain used. Further work is needed to investigate this.</td>
</tr>
<tr>
<td>How should a reference preparation for use in a serological assay be calibrated?</td>
<td>Calibration of standards should be done using a multiple dilution assay with a</td>
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<td>Question/Comment</td>
<td>Response</td>
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<tr>
<td>functional end point (e.g. challenge test or serological test with titration of immune sera by toxin neutralisation test).</td>
<td></td>
</tr>
<tr>
<td>When should the test for degree of adsorption be performed?</td>
<td>The WHO recommendations state that the test should be performed at least on the final lot – it may also be performed at the final bulk stage (but can be omitted if performed on the final lot).</td>
</tr>
<tr>
<td>Can a serological assay be used to measure the potency of diphtheria and tetanus components in the same vaccine?</td>
<td>Yes – although it is necessary to demonstrate a suitable dose response to both components in validation studies. For some products, the potency difference may be too great to allow both components to be assessed in the same animals. In some cases, it may be necessary to increase the number of vaccine dilutions to five or six to ensure that a valid dose response range is covered for both components. It may be difficult to do this in mice because the potency of the tetanus component (particularly in wP combinations) could be extremely high compared to the diphtheria component. Validation studies are needed on a product-by-product basis.</td>
</tr>
<tr>
<td>Should secondary reference preparations be homogenous with respect to the product under test?</td>
<td>The principle of biological standardisation is, that like is compared with like – use of a product specific reference preparation is as close to homogeneous as possible and should produce consistently parallel dose response curves. However, use of product specific reference preparations should be carefully considered for the following reasons: 1. stability must be closely monitored since these are likely to be liquid preparations; 2. shelf life is relatively short (compared to a freeze-dried standard) and replacement batches need to be calibrated at frequent intervals; 3. NCLs may need to test different products from different manufacturers and the use of many different reference preparations can be</td>
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<td>Question/Comment</td>
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<tr>
<td>How can trend analysis be performed where a single dilution assay is used?</td>
<td>A truly quantitative estimate of potency is not obtained where a single dilution assay is used – therefore, it is not possible to monitor the potency of the product over time. Where a serological assay is used, it is possible to monitor antibody titres for the reference vaccine and test vaccine and these can be monitored over time to assess batch-to-batch consistency for the product and stability of the reference vaccine.</td>
</tr>
<tr>
<td>Clarification on the requirements for the specific toxicity test (when animals die from non-specific causes) for D and T was requested.</td>
<td>Any animals that die during the test must undergo necropsy to rule out diphtheria or tetanus intoxication as the cause of death. If only one dies from non-specific causes then the vaccine passes the test. If more than one animal dies from non-specific toxicity then a re-test is performed and four out of five animals must survive the test – if one animal dies then the cause of death must be shown to be non-specific in order for the vaccine to pass. If more than one animal dies in the re-test (even from non-specific causes) then the vaccine does not comply.</td>
</tr>
<tr>
<td>The option to omit the innocuity test on the final lot was discussed.</td>
<td>For D and T vaccines, the innocuity test on the final lot can be omitted once consistency in production has been demonstrated (and only with the approval of the NRA). This is because the test for specific toxicity performed on the final bulk is much more sensitive than the innocuity test performed on the final lot. However, it was also acknowledged that for combined vaccines the situation may be more complex and that the innocuity test should be retained – at least for a defined percentage of the final lots.</td>
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<tr>
<td>The use of the Vero cell toxicity test was discussed particularly with respect to the relative sensitivity of the guinea pig assay and cell culture assay.</td>
<td>The Vero cell assay is approximately 1000 times more sensitive than the lethal challenge method in guinea pigs and approximately 20 times more sensitive than the intradermal challenge method in guinea pigs.</td>
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pigs. The dose of toxoid used in the Vero cell assay ensures that the assay is at least as sensitive (if not more so) than the in vivo models. However, the sensitivity of Vero cells to diphtheria toxin decreases with increasing passage number and it is important to include a positive control toxin in every assay to monitor sensitivity over time. At NIBSC, the same batch of cells is not used for toxicity testing beyond passage 20. A defined limit for the passage number should be established in validation studies.

### Quality control methods for whole cell pertussis vaccines

Dr Kevin Markey (NIBSC) discussed the critical aspects of the potency, safety and identity tests for whole pertussis that are currently included in the WHO Recommendations for whole cell pertussis vaccines (wP), and WHO Manual for DTP vaccines. There are a number of methods included in these WHO documents but not all were discussed here.

In terms of wP potency the intra-cerebral mouse protection assay was discussed with particular emphasis on the critical factors of the assay. This has a predictive value for the protective activity of the vaccine in man and estimates the potency of pertussis vaccines on their ability to protect mice against intra-cerebral challenge with virulent *Bordetella pertussis*. The potency is expressed in IU calculated by comparing the effective dose of the test vaccine to a reference vaccine. Some of the critical factors include the challenge *B. pertussis* strain which must be in Phase I growth to ensure virulence, and the challenge suspension should be 100 – 1000 times the LD$_{50}$. The mouse strain used should be sensitive to *B. pertussis* challenge and be fully validated. Also of importance is the choice of reference vaccine. For this the International Standard for wP vaccine, or other national/ in-house reference preparation calibrated in terms of IU should be used as the working reference. The doses of test and reference vaccine used in the assay should give a dose-response, linear range and parallelism. Details on assay validity pass criteria and data monitoring were also presented.

The mouse weight gain test (MWGT) is used to assure the safety of wP vaccines and it is based on the ability of certain toxins or components from *B. pertussis* to cause weight loss in young mice. A number of toxins in wP vaccines may induce weight loss and previous studies have indicated that there is a correlation between MWGT with adverse reactions in children. In the test the weight gained by mice vaccinated with the test vaccine is compared to the weight gained by mice immunised with PBS. A reference vaccine may also be included. Of importance in this test is the initial weight of the mice – they should be small enough to gain...
weight, measurements should be made at the same of day and excess weight gain may be the result of ascites. Identity tests are required as it’s vital that wP vaccines contain *B. pertussis* strains expressing 1, 2 and 3 serotypes. Three common assays; slide agglutination, ELISA and immunodiffusion assays were discussed. All require the detection of Fimbrae 2 and 3 antigens by monoclonal antibodies or anti-sera. The key points in reviewing manufacturer’s protocol were also presented. This session was followed by a short discussion and the main questions/comments are summarised in Table 5.

Table 5. Summary of round table discussion – Laboratory testing of whole cell pertussis vaccines

<table>
<thead>
<tr>
<th>Question/Comment</th>
<th>Response</th>
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<tbody>
<tr>
<td>The point at which the colony forming units of the challenge suspension of <em>B. pertussis</em> in the intra-cerebral mouse protection assay was discussed.</td>
<td>Some laboratories use growth curves based on optical density prior to challenge to ensure correct bacterial density. Other labs also determine the CFU of the challenge bacterial suspension before and after challenge has been performed. This is something to address in method validation.</td>
</tr>
<tr>
<td>The upper limits of the intra-cerebral mouse protection assay were discussed.</td>
<td>There are no upper limits and TRS 941 states that these should not be less than 4.0 IU per single human dose with a lower fiducial limit of the estimated potency being not less than 2.0 IU. However, the small print states that “in some countries and upper limit is also specified”. But anyhow the consistency of the vaccine shall be monitored and there shall not a significant deviation.</td>
</tr>
<tr>
<td>There was a query in relation to how parallel line analysis is performed.</td>
<td>Parallel line assay is achieved by probit analysis.</td>
</tr>
<tr>
<td>The MWGT was further discussed in relation to weight gains of more than 150% which may indicate the presence of ascites.</td>
<td>TRS 941 states that if the average weight gain per mouse in the vaccine group is greater than 150% of that per control mouse, ascites production should be suspected and the test should be considered invalid.</td>
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</table>

**Quality control methods for acellular pertussis vaccines**

Dr Markey (NIBSC) discussed the critical aspects of the safety, immunogenicity and identity tests for acellular pertussis vaccines (aP) that are currently included in the WHO Recommendations for aP and DT based combination vaccines. There are a number of methods included in these WHO documents but not all were discussed here.
The Histamine sensitisation test is used as a safety test for aP. The principle of the test relies in the fact that mice exposed to active pertussis toxin (PTx) exhibit sensitivity to histamine challenge leading to anaphylactic shock, temperature drop and death. It can also be used to assess reversion to toxicity. It is the only generally accepted test for PTx in final bulk/product and currently two variants of the assay are in use; pass-fail method (lethal end point) and quantitative method (temperature measurement). Factors discussed included assay validation, validity criteria, specifications, mouse strain, reference toxin calibration and outstanding issues associated with the assay.

The Mouse Immunogenicity Test (MIT) measures the antibodies against aP antigens generated by mice after they have been immunised with test vaccine and controls. It is a routine QC procedure to monitor consistency of production and is not a protective potency assay. A number of critical factors were discussed such as mouse strain, determining the linear-response region of the dose-response curve, specifications, the importance of a stable reference serum, the ELISA method and reference vaccine.

Animal protection models may also be used for aP control. The advantages and disadvantages of some common tests such as the modified intra-cerebral challenge assay (MICA), intra-nasal challenge model and aerosol challenge assay were discussed.

Identity assays to determine the presence of aP antigens by sandwich ELISA (or immunoblotting) was also discussed along with the key points in reviewing manufacturer’s protocol. This session was followed by a short discussion and the main questions/comments are summarised in Table 6.

Table 6. Summary of round table discussion – Laboratory testing of acellular pertussis vaccines

<table>
<thead>
<tr>
<th>Question/Comment</th>
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<tbody>
<tr>
<td>There was a discussion on the reference serum used in MIT and whether it is product specific.</td>
<td>The reference serum is not product specific and should be calibrated in IU. WHO Reference Reagent <em>Bordetella pertussis</em> anti-serum (mouse) 1RR (NIBSC code: 97/642) can be used.</td>
</tr>
<tr>
<td>Further information on antigens for coating ELISA plates for MIT was requested.</td>
<td>It was emphasised that it is important to use non-detoxified antigens in coating ELISA plates.</td>
</tr>
<tr>
<td>Questions regarding aP working standards for immunogenicity test.</td>
<td>Standard reagents for immunogenicity are available from NIBSC.</td>
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Statistical analysis of vaccine potency assays
Combination of assay results
Mr Peter Rigsby, NIBSC presented an overview of the statistical methods used for the analysis of vaccine potency assays. This covered some general points regarding assay design, details of the parallel-line model used for quantitative assay data, the probit model used for quantal assay data, the methods used to assess assay validity and interpretation of results. The topics were illustrated using examples of assays included in the WHO Manual for Quality Control of Diphtheria, Tetanus and Pertussis Vaccines. This was followed by a presentation regarding the combination of results from independent assays, covering the assessment of homogeneity of potency estimates and choice of appropriate mean. Copies of the presentation slides were distributed to all participants. The main questions/comments are summarised in Table 7.

Table 7. Summary of round table discussion – Statistical analysis of vaccine potency assays & Combination of assay results

<table>
<thead>
<tr>
<th>Question/Comment</th>
<th>Response</th>
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<tbody>
<tr>
<td>The balance and randomisation of assays is important.</td>
<td>As an example, it was recommended that the order in which samples are loaded on a microtitre plate is randomised, unless shown to have no effect on response. Ideally this should be addressed during method validation.</td>
</tr>
<tr>
<td>How can one ensure that the 20-80% response range is covered by the dilutions used in the assay?</td>
<td>It is important that the dose response covers a large part of the linear range in order to demonstrate regression and an appropriate range of dilutions should be determined during validation or development studies.</td>
</tr>
<tr>
<td>Can more than one test sample be analysed at the same time?</td>
<td>Multiple samples in a single assay can be analysed against the reference at the same time but the overall common slope may not be valid for one sample and therefore affect the other samples. Therefore, it is recommended to analyse each test sample individually. The method of analysis used (including choice of transformation) should be used consistently for all assays.</td>
</tr>
<tr>
<td>Can statistical tests be applied to detect outliers?</td>
<td>Statistical methods can be used to identify outliers but these should only be excluded with clear reasons/justification.</td>
</tr>
<tr>
<td>How many parameters are estimated in the parallel line model?</td>
<td>Four and five parameter models are used for sigmoid dose-response curves. Responses in the linear region are calculated based on two parameters; the slope and the intercept (parallel-line model).</td>
</tr>
<tr>
<td>When can an assay be changed to single</td>
<td>When changing from an assay that uses...</td>
</tr>
</tbody>
</table>
**Question/Comment** | **Response**
---|---
dose only? | multiple doses to single dose, consistency should be demonstrated in the multiple dose assays. These assays should also show that the dose of the reference is at the lower end of the linear range. Data monitoring is important to detect any change in the reference, although the type of monitoring performed will depend on the response type (e.g. challenge vs. serology).

Discussion on increasing the number of dilutions in an assay. | A current assay has four dilutions but may have to increase to six and this was considered acceptable.

Do dilutions need to be equally spaced/same for test and reference vaccines? | It is recommended to use the same spacings for dilutions of reference and test vaccines (assuming that they are expected to be parallel) but the software used for analysis should be able to deal with any dilution spacings.

How many data points should overlap for the test and reference? | There are no set requirements on the number of data points that should overlap and situations where the ranges of the test and reference don’t overlap should be dealt with on a case-by-case basis. SOPs should contain limits to ensure samples overlap in quantitative assays.

What if the 50% value is not covered when using probit analysis? | If the 50% value in probit analysis is not covered (i.e. the ED50 is not within the range of dilutions tested) then the assay should be repeated.

How should the LD$_{50}$ of toxin be calculated? | Probit analysis or the Spearman-Kärber method can be used.

Should homogeneity be determined every time a valid test is repeated? | Yes, this is the case even if the second test uses a larger group of animals. Use of the weighted geometric mean is only justified when homogeneity has been demonstrated.

**Trend analysis of data for lot release**

**Tools for data monitoring**

**Practical consideration for trend analysis**

Mrs Jivapaisarnpong and Mr Rigsby gave a series of presentations on trend analysis and data monitoring covering protocol review, trend analysis, comparison of results with
manufacturer and the construction and use of control charts. This was followed by a round table discussion and the main questions/comments are summarised in Table 8.

Table 8. Summary of round table discussion – Trend analysis & Data monitoring

<table>
<thead>
<tr>
<th>Question/Comment</th>
<th>Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Should the manufacturer’s summary protocol data be reviewed and subjected to trend analysis?</td>
<td>The same data monitoring approaches can be applied to data reported by the manufacturer in the summary lot protocol.</td>
</tr>
<tr>
<td>When an NCL plots data from a manufacturer the NCL is responsible for setting up limits but close collaboration with the manufacturer is also required because they should also be involved in selecting limits. The NCL should detect any shifts in values.</td>
<td></td>
</tr>
<tr>
<td>In situations where manufacturers import bulk, fill it and then export, the NRA that releases the vaccine should be consulted if a problem arises regardless of where the vaccine was produced.</td>
<td></td>
</tr>
<tr>
<td>Should log values be used for data monitoring?</td>
<td>The log transformed values should be used where appropriate for data monitoring e.g. for relative potencies or ED50’s.</td>
</tr>
<tr>
<td>How many data points are required to set up control limits?</td>
<td>It is recommended that a minimum of twenty data points should be used to generate limits, but should use as many as possible and trend analysis should be an on-going process.</td>
</tr>
<tr>
<td>What action should be taken when the results obtained by a NCL differ significantly to those from a manufacturer?</td>
<td>In such situations the methods used by each lab should be analysed to identify any reasons for the difference. NRA can request the manufacturer to change their assay.</td>
</tr>
<tr>
<td>Can NCL testing be reduced?</td>
<td>An NCL may reduce testing if consistency is demonstrated through trend analysis (or increased if inconsistency is observed).</td>
</tr>
</tbody>
</table>

Round table discussion involving all participants

The chair invited each participant to raise any additional questions related to any of the topics covered during the workshop. A summary of the questions/comments and responses is shown in Table 9.

Table 9. Summary of final round table discussion
<table>
<thead>
<tr>
<th>Country</th>
<th>Comment/Discussion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bangladesh</td>
<td>Pointed out that statistics for trend monitoring may be helpful.</td>
</tr>
<tr>
<td>China</td>
<td>The current national reference for tetanus potency has similar potency (400 IU) in both mice and guinea pigs. When establishing a new national reference it will have to be calibrated against the International Standard in both mice and guinea pigs using the appropriate assigned value for each model. A conversion factor may be needed if a significant shift in potency estimates for a product is observed.</td>
</tr>
<tr>
<td>India</td>
<td>Discussion on lack of upper limits for DT highlighted that none have been set because clinical trials indicated that higher content had no effect plus potency is just an estimate influenced by a number of factors. It was pointed out that consistency should be monitored. It was found in Japanese clinical data that there was a correlation between guinea pig potency and antibody titre in infants.</td>
</tr>
<tr>
<td>Indonesia</td>
<td>What are the specifications for low antigen content diphtheria? No defined specifications and depends on NRA. The WHO recommendations for diphtheria vaccines contain a small print statement “In some cases it is recommended that the lower 95% confidence limit of the estimated potency of diphtheria vaccines intended for boosting should not be less than 2 IU/SHD”. Discussion on release certificates from countries in which the vaccines are not manufactured highlighted that there is mutual recognition between OMCLs in the European Union (EU) and vaccines may be registered in countries other than where they are manufactured. It was also pointed out that it’s mandatory for the manufacturer to perform all the required testing and take responsibility for the quality of the product. It was also pointed out that expiry dates have to be approved by NRA.</td>
</tr>
<tr>
<td>Iran</td>
<td>Problems with guinea pig supply mean that calibration of local standards is difficult. The question of whether an independent laboratory, such as NIBSC, could perform potency assays for calibration was discussed. This would need to be considered on a case by case basis.</td>
</tr>
<tr>
<td>South Korea</td>
<td>The 20 batches for setting trend analysis criteria is only a rule of thumb but can use whatever number of batches available. There are no limitations for LAL tests in wP vaccines because the levels are so high but levels should be monitored for consistency.</td>
</tr>
<tr>
<td>Myanmar</td>
<td>Discussion on testing Hib component and the fact that an ELISA assay has replaced the in vivo assay.</td>
</tr>
<tr>
<td>Malaysia</td>
<td>No further comments but asked for help when they set up their new laboratory.</td>
</tr>
<tr>
<td>Pakistan</td>
<td>What action should be taken in relation to market authorisation when the volume of batch size for filling is changed? It was agreed that there should be a validated minimum and maximum capacity for fermenters but is should be acceptable to reduce the volume</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Country</th>
<th>Comment/Discussion</th>
</tr>
</thead>
</table>
| Philippines | Does a country that receives pre-qualified vaccines from WHO/UN agency need to perform lot release?  
Dr Lei, WHO – no as it will have gone through lot release already.                                                                                                                                                                                                                       |
| Sri Lanka | Noted that, when establishing control limits, some previous batches were found to be outside these limits.  
Control limits are not applied retrospectively i.e. to reject batches that were tested before the limits were established but are used to see if subsequent batches are outside of the limits once they have been established.                                                                                         |
| Thailand | Clarification was requested on calculating geometric mean for trend analysis. It was clarified that, for log normally distributed data (e.g. potencies), convert to log and perform the calculation. At the end convert back from log to get unit values.                                                                                      |
| Vietnam  | Diphtheria potency testing in mice and the issue of transferability was discussed. It was acknowledged that many laboratories experience significant difficulties complying with WHO recommendations for diphtheria vaccines because they cannot obtain a suitable supply of guinea pigs to perform transferability studies (particularly in the knowledge that this needs to be done for each product). NIBSC will work with WHO to investigate the possibility of assigning units to the current IS based on calibration in mice – but this issue is unlikely to be resolved in the short term. |

**Introduction, demonstration and use of Bioassay Assist**

Dr Yoshinobu Horiuchi and Dr Masaki Ochiai, National Institute of Infectious Diseases (NIID), Japan provided an introduction and demonstration of the statistical analysis software Bioassay Assist which can be used for statistical analysis (parallel line and probit models) and some data monitoring. Participants were able to gain some experience in use of the software by running several examples on their laptops. Calculation of weighted mean of repeated assay results, covering the assessment of homogeneity of the estimates, using Excel was also presented.

**Conclusions**

The workshop provided an opportunity for representatives of NCLs and/or NRAs from countries of the South-East Asia and Western Pacific Regions to learn about recent revisions to WHO recommendations for diphtheria, tetanus and DT-based combined vaccines. An overview of methods used in laboratory testing of diphtheria, tetanus and pertussis vaccines, as described in the *WHO Manual for Quality Control of Diphtheria, Tetanus and Pertussis Vaccines*, was also provided. The workshop provided an opportunity for participants and facilitators to share experience and knowledge to help build capacity in regulatory oversight for DTP vaccines.
Appendix 1. List of participants
Facilitators:
Dr Yoshinobu Horiuchi, Divison of Quality Assurance, National Institute of Infectious Diseases (NIID), Tokyo, Japan. Mrs Teeranart Jivapaisarnpong, Institute of Biological Products, Department of Medical Sciences, Ministry of Public Health, Thailand. Dr Kevin Markey, Division of Bacteriology, National Institute for Biological Standards & Control (NIBSC), Blanche Lane, South Mimms, United Kingdom. Dr Masaki Ochiai, Department of Quality Assurance and Radiological Protection, NIID, Tokyo, Japan. Mr Peter Rigsby, Biostatistics Group, NIBSC, Blanche Lane, South Mimms, United Kingdom. Dr Paul Stickings, Division of Bacteriology, NIBSC, Blanche Lane, South Mimms, United Kingdom.

Participants:
Mr Mohammad Alauddin, National Control Laboratory, Mohakhali, Dhaka, Bangladesh. Mr Assajun Amen, Institute of Biological Products, Department of Medical Sciences, Ministry of Public Health, Nonthaburi, Thailand. Mr Abdul Samad Khan, Ministry of National Health Services, Regulations and Coordination Drug Regulatory Authority of Pakistan, Pakistan. Mr Kwan Soo Kim, National Center for Lot Release, National Institute of Food and Drug Safety Evaluation, Ministry of Food and Drug Safety (MFDS), Chunchonbuk-do, Republic of Korea. Mr Suresh Kumar, Central Drugs Laboratory, CRI- Kasauli, Himachal Pradesh, India. Mrs Dede Kusmiaty, National Agency of Drug and Food Control, National Quality Control Laboratory of Drug and Food, Jakarta, Indonesia. Dr Chulhyun Lee, National Center for Lot Release, National Institute of Food and Drug Safety Evaluation, Ministry of Food and Drug Safety (MFDS), Chungcheongbuk-do, Republic of Korea. Ms Do Khanh Linh, National Institute for Control of Vaccine and Biologicals, Ha Noi, Viet Nam. Jose Marie Anthony Mampusti, Research Institute for Tropical Medicine, Muntinpula City, The Philippines. Dr Saeed Reza Pakzad, Food and Drug Control Laboratories (FDCL), Iran. Dr Nasima Pervin, National Control Laboratory, Dhaka, Bangladesh. Dr Peng Luo, National Institutes for Food and Drug Control, Beijing, China. Mrs K A Devika N. Perera, Dpt of Rabies & Vaccine Quality Control, National Control Laboratory, Medical Research Institute, Colombo, Sri Lanka. Mrs Ratih Pujilestari, National Agency of Drug and Food Control, National Quality Control Laboratory of Drug and Food, Jakarta, Indonesia. Ms Caren M Recto, Food and Drug Administration, Muntinpula City, The Philippines. Ms Suchita Sharma, Central Drugs Laboratory, Himachal Pradesh, India. Mr Apichai Supasansatorn, Institute of Biological Products, Department of Medical Sciences, Ministry of Public Health, Nonthaburi, Thailand. Mrs Sri Wahyunngsih, National Agency of Drug and Food Control, National Quality Control Laboratory of Drug and Food, Jakarta, Indonesia. Dr Nwe Ni Win, National Health Laboratory, Yangon, Myanmar. Dr Yajun Tan, National Institutes for Food and Drug Control, Beijing, China. Ms. Le Thi Hoang Yen, National Institute for Control of Vaccine and Biologicals, Ha Noi, Viet Nam. Wan Noraini Binti Wan Yussof, National Public Health Laboratory, Ministry of Health Malaysia Lot 1853, Selangor, Malaysia.

Observers: Dori Ugiyadi, BioFarma, Indonesia. Tri Yuni Nugraha, BioFarma, Indonesia
WHO Secretariat:
Dr Dianliang Lei, Technologies, Norm and Standards (TSN), Department of Essential Medicines and Health Products (EMP), Health Systems and Innovation (HIS), World Health Organization, Geneva, Switzerland. Mrs Deasy Tomatala, WHO Country Office, Indonesia. Gedung Dr. Adhyatma, Ministry of Health, Jakarta, Indonesia.

NADFC Secretariat:

Authors:
Dr Paul Stickings, Peter Rigsby, and Dr Kevin Markey, National Institute for Biological Standards and Control, UK.
Dr Dianliang Lei, World Health Organization, Switzerland.
On behalf of the WHO Workshop on Laboratory Testing of DTP Combined Vaccines.