EXPERT COMMITTEE ON BIOLOGICAL STANDARDIZATION  
Geneva, 21 to 25 October 2013

WHO Consultation on Commutability of WHO Biological Reference Preparations for *In Vitro* Detection of Infectious Markers  
WHO Headquarters, Geneva, 18-19 April, 2013

Meeting report

NOTE:

This report aims to inform the Expert Committee on Biological Standardization 2013 with regard to the general consensus of the WHO consultation on the above subject.  
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**Rapporteurs:**

Dr. Jay Epstein (CBER, FDA)
Dr. Micha Nübling (PEI)
Dr. Chris Burns (NIBSC)
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Opening Remarks

The meeting was opened by Dr. Ana Padilla who welcomed the participants to WHO on behalf of Mr. Kees de Joncheere, Director EMP, WHO/HQ. Dr. Padilla presented the background to the meeting, making reference to the core mandate of the WHO in the preparation of WHO international standards in conjunction with the WHO collaborating centers. Specific reference was made to the public health importance of standards for calibrating diagnostic assays for the in vitro detection of infectious markers. It was explained that the focus of the meeting would be an evaluation of the options for assessing the commutability of WHO reference preparations in the area of infectious markers. Dr. Padilla provided an outline of the objectives and expected outcomes of the following two days discussion. In line with WHO procedures, Dr. Harvey Klein was proposed as meeting chairman and representatives from three WHO collaborating centers as meeting rapporteurs, Dr. Jay Epstein (CBER, FDA), Dr. Micha Nübling (PEI) and Dr. Chris Burns (NIBSC). Hearing no objections, these proposals were agreed on.

Session 1: WHO Biological Reference Preparations and Commutability – An Overview

Introduction

Dr. Epstein introduced the topic of commutability and explained the clinical importance of harmonization of in vitro assays for infectious disease markers to facilitate the evidence-based derivation of clinical guidelines used in the diagnosis, treatment and monitoring of patients. One of the goals in the harmonization of in vitro measurements is to assure that the same numeric result for a measurand, which could be an antigen, antibody or nucleic acid, will be obtained in a patient sample irrespective of the assay method used to derive that result. A lack of harmonization can lead to different methods providing divergent results for the same clinical sample and clinicians and other healthcare professionals, who are often unaware of these differences, may wrongly classify a patient’s health status. However, the difficulties associated with the preparation of reference materials for complex biological measurands were recognized. These included their inherent complexity and biological variability, the frequent requirement to pool patient samples, possible purification of the measurand from its native matrix or the substitution of the native measurand with a non-native version (e.g. recombinant or cell culture-derived) and the processing steps that are often required to ensure their long term stability. These alterations clearly mean that the reference standard may be different from the native clinical sample and this is very important when considering the likely commutability of reference materials. Following on from this, commutability was defined as:

“.....the equivalence of the mathematical relationships between the results of different measurement procedures for a reference material and for representative samples from healthy and diseased individuals.”

Dr Epstein then briefly introduced the concept of multicenter international collaborative studies for the characterization and assessment of the suitability of WHO biological reference preparations. It was recognized that there were limitations on what information could be obtained in these studies and that this would be an important topic for further discussion during the course of the meeting.
The primary question raised was, given the obvious limitations, were additional studies needed to better characterize the commutability of WHO biological reference preparations for the in vitro detection of infectious markers, and, if so, what strategies were feasible and most appropriate.

**The Clinical Laboratory Perspective and AACC Harmonisation Initiative**

Dr Greg Miller from the American Association for Clinical Chemistry (AACC) and the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) outlined the importance of commutable reference materials from the perspective of the wider clinical chemistry and laboratory medicine community. Laboratory test results are used to inform decisions on diagnosis and treatment of diseases and it is important that results from different measurement procedures give consistent and comparable results so that clinical practice guidelines can be correctly applied to patient care decisions. The ISO document 17511:2003 provides a standard for traceability of calibration of routine clinical laboratory procedures to higher order reference materials and reference measurement procedures. Such calibration traceability has the potential to achieve consistent and comparable results that are sustainable over time and among different measurement procedures.

Reference measurement procedures are not available for a large number of measurands of clinical importance. In these situations, it is desirable to have a reference material that can be used as a common calibrator with the desired goal to provide comparable results among different routine clinical laboratory procedures. Unfortunately, many reference materials in current use are not commutable with clinical samples. Consequently, when such reference materials are used as common calibrators, the routine measurement procedures do not give comparable results for patient samples. Thus, incorrect diagnosis or treatment decisions may be made. The clinical laboratory profession must include validation of commutability of reference materials as a requirement to demonstrate that they are suitable for their intended use.

In 2010, AACC convened a conference of international stakeholders to discuss a global response to the problem of lack of harmonization of clinical laboratory test results with a focus on situations when there was no reference measurement procedure. Participants recommended the creation of an International Consortium for Harmonization of Clinical Laboratory Results that would: provide a process to prioritize measurands to be harmonized, determine what technical gaps existed to harmonize a given measurand, address technical approaches to achieve harmonization when reference procedures are not available, and provide an information portal for all standardization or harmonization activities being conducted by different organizations worldwide. AACC is currently working with its domestic and international partners to implement this recommendation (see [www.harmonization.net](http://www.harmonization.net)).

**Specific Challenges in the Assessment of Commutability for WHO Reference Materials**

Dr Chris Burns from the National Institute for Biological Standards and Control (NIBSC) provided an outline of the scope of the WHO Biological Standards programme at NIBSC. This programme includes, within the infectious disease area, a number of important examples where WHO reference
materials have been developed for the quantitation of antibody, antigen and nucleic acids, which have promoted global harmonisation of assays. Examples include WHO reference materials for blood borne viruses such as hepatitis B and C, and a recently established WHO reference material for cytomegalovirus, an important agent in transplantation, where rising or threshold levels of virus in the blood may lead to chemotherapeutic interventions. The calibration of these assays is therefore important to guide clinical practice.

Given that the majority of assay methods used in laboratory medicine are designed to perform measurements directly in native clinical samples (e.g. serum, plasma or urine), the most obvious source of material for the preparation of commutable reference standards is patient-derived samples. However, it is often impossible or, in some cases unethical, to obtain sufficient material from a single patient to allow the preparation of a reference material, particularly a WHO reference material which is usually prepared in a reasonably large number of ampoules or vials. As a result, the preparation of such a reference material may require the pooling of patient samples or the purification of the measurand from its native matrix. These alterations result in a reference standard which may be different from the native clinical sample. In addition, this is compounded by the requirement to ensure the long term stability of reference materials. Long term stability is particularly important for WHO reference materials, since in most cases, they represent the highest order standard available and are used to calibrate a range of secondary reference materials. As a result, it is important to avoid the regular replacement of these reference standards and the associated potential for discontinuity of unitage. To ensure this long term stability, WHO reference materials are lyophilised and/or potentially further altered in comparison to the native specimen matrix, for example by the addition of stabilizing excipient(s). It is therefore important to determine whether such alterations affect the results of assay methods in a way that renders the reference standard unfit for the purposes of establishing calibration traceability and assay harmonisation.

Dr Burns summarised the factors that should, ideally, be considered in a study designed to fully investigate the commutability of a reference material. These include the inclusion of all available methods for which the reference material will be used and the inclusion of a suitable number of representative (normal and diseased) clinical samples spanning the appropriate range of values for the measurand. These clinical samples should be screened to exclude any that contain substances that are known to interfere with one or more methods in the study. Although pooling or freezing clinical samples should be avoided, if this is necessary for logistical reasons, then the effect of these alterations should be assessed prior to the study. Importantly, laboratories in the study should be prequalified to exclude those with unusually high imprecision. These study design considerations may not always be the same as those in a traditional WHO collaborative study where the primary intention is to demonstrate that a reference material is “fit for purpose” as far as practical, and in most cases to assign a value to the reference by comparison with existing reference materials where available. A demonstration that the use of the candidate reference will improve agreement between laboratories for other test samples is critical for the candidate to be accepted as a WHO reference material and these test samples may include materials other than clinical samples, for instance, the unprocessed bulk used to make the reference preparation. In addition, laboratories in the study are not prequalified. In fact, the study is purposely designed to include a large number of laboratories which represent different types of end-user, giving good representation geographically and including a wide range of different assay types. Furthermore, resource limitations often restrict the size of the study and in some cases also the number of test samples and the range of assays included in the study. These restrictions may limit the information that can be obtained from the study.
Given these recognised difficulties, Dr Burns summarised the options for assessing commutability outside of the traditional WHO collaborative study approach, with examples that were to be discussed later in the meeting. These included a separate commutability study (WHO International Standard for cytomegalovirus), with an External Quality Assurance (EQA) scheme (WHO International Standard for Diphtheria Antitoxin, Human) and in partnership with other organisations such as IRMM, IFCC or AACC.

All these conclusions are made with the assumption that all assays are well designed and fit for their intended purpose, e.g. consistent and reliable quantitation of the measurand. However, design differences between assays, particularly specificity for the measurand, may contribute to variability of results and it may be these differences, rather than non-commutability of the reference material, that are the cause of the variability.

**Statistical Approaches to Assessing Commutability**

Mr. Alan Heath from NIBSC gave an overview of the statistical approaches that are used to assess the commutability of reference materials and highlighted some limitations of these approaches. In addition, limitations in the current approach adopted within WHO collaborative studies were also discussed, the most critical being the lack of genuine patient samples in many studies. Any statement on commutability of a reference material is limited to the assay methods and samples studied. It is not a generic property of a reference material, and it is not possible to simply state that any reference material is “commutable”. The definition of commutability has to be assessed against a background of assay variability, and heterogeneity between clinical samples. The definition is often interpreted as a statistical assessment of “Do the results for the reference material fall within the general distribution of results from a range of clinical samples, when different assay methods are applied?”. This approach gives an arbitrary ‘yes’ – ‘no’ assessment which is dependent on the statistical methods used, and the degree of assay variability. It does not give a quantitative measure of how effective the reference will be at reducing inter-method differences.

When multiple methods are being studied, a large number of method-pair comparisons can be made, which may result in a mix of “commutable” and “non-commutable” classifications, which becomes hard to interpret. Some multivariate approaches have been proposed, to give an “overall” assessment, but again the interpretation of these results is not always clear.

The WHO collaborative studies conducted to establish reference materials use a different approach to analysis. Where possible, a small number of additional study samples are included along with the candidate reference materials, ideally representative of the type of material that the reference will be used with. The extent by which the inter-method and inter-laboratory variability is reduced by the use of a reference material can be quantified for these samples, and a judgement made on how useful the reference material will be. This is a pragmatic approach that even if a reference material is not fully commutable by a strict metrological definition, it may still be of value. The main limitation with this approach is the lack of genuine clinical samples included in these studies. It may also be possible to assess potential causes of non-commutability, such as material processing steps, in smaller scale pilot studies.
An example of the WHO study approach is the recent study to establish an International Standard for CMV for NAT assays (details below). This study included a freeze-dried candidate standard, along with the starting liquid bulk material, a second freeze-dried material of a different virus strain, and a purified cloned DNA preparation. Using the candidate standard led to a reduction in inter-laboratory variability for potency estimates of the sample of a different strain. Using the purified DNA material as a reference did not lead to any reduction in inter-laboratory variability for the other study samples.

Future aims are to improve the statistical definition and assessment of commutability to remove the dependence on assay variability, and to provide a measure of commutability that can be related to improvement in agreement between laboratories for panels of clinical samples. Consensus on how to interpret results from multiple method studies is also required.
Session 2: Reference Preparations for NAT assays

Prior to the presentation of individual case studies, Dr Micha Nübling from PEI provided an overview of the history and purpose of WHO International Standards (ISs) designed for harmonization of Nucleic Acid Amplification Technology (NAT) based assays. The “infectious markers” NAT standards cover DNA or RNA of pathogens, e.g. viruses or bacteria. One of the major considerations when thinking about the commutability of reference materials in this area is the assumption that target analytes of NAT assays might be assumed as quite well-characterized (e.g. detailed nucleic acid sequence information) and as a result standardization or harmonisation might be considered as easily feasible. However, the analyte is embedded in protein structures and therefore has to be purified prior to amplification. Extraction of nucleic acids is a complex enterprise and may contribute a major part to the variation of the whole NAT procedure. Furthermore, the target analyte in patient or blood donor specimens may consist of sequence variations, e.g. viral strains or genotypes, while the WHO IS is usually prepared from one well-characterized material (e.g. patient plasma, cell culture) representing only one variant, strain and genotype. Another additional complication is whether the matrix used for the WHO IS itself (e.g. plasma) is different compared with the matrices for which the assay may be used, e.g. urine or cerebrospinal fluid (CSF). Therefore, different aspects need to be considered when assessing potential commutability issues of WHO ISs for NAT assays. The current design process for NAT standards was reviewed. The question was raised whether potential effects introduced into NAT standards by inactivation or lyophilization steps are already adequately addressed with current approaches. It was thought that pilot studies might be an important future goal for new standardization projects in this field. WHO ISs for NAT were reviewed in regard to potential differences or alterations introduced between the first and the subsequent replacement standard(s) by measures which may include changing to a different variant/genotype, introduction of inactivation methods, the presence or absence of specific antibodies, a new source of material (e.g. cell culture instead of patient). Although any such potential differences between first and subsequent (replacement) WHO ISs were always presented and discussed in expert forums, for example SoGAT (Standardization of Genome Amplification Techniques) meetings, it was recognized that they should be avoided as far as possible. However, the experience gained in the NAT field shows convincing evidence that most of the biological standardization projects for NAT markers have been success stories, and the vast majority of these standards appear to be fit for purpose. Nevertheless, it was noted that an improvement in the procedures used to manufacture these reference materials might be warranted, for example, an investigation of the potential effects of freeze-drying or other manipulations, early on in the lifetime of a project - perhaps in pilot studies – could be performed by the responsible WHO Collaborating Center. In addition, where possible a more direct assessment of the commutability of these reference materials with clinical samples would also be required. These issues were discussed in more detail in the presentations of individual case studies (briefly summarized below and in more detail in Appendix 1).
1st WHO IS for Human Cytomegalovirus (HCMV) for Nucleic Acid Amplification (NAT)-Based Assays and its commutability

Dr Jacqueline Fryer (NIBSC) described the establishment of the WHO IS for HCMV for NAT assays and the evaluation of its ability to harmonise CMV NAT-based assays. The final WHO IS candidate preparation (Merlin strain, lyophilized) was formulated with universal buffer to be spiked into different patient matrixes (plasma, whole blood, CSF, urine etc). The collaborative study included the liquid counterpart of the WHO IS candidate, a liquid cell culture derived AD169 strain and a bacterial artificial chromosome (BAC) with the complete viral genome of the Merlin strain. The collaborative study showed pronounced harmonization of assays by the WHO IS candidate with regard to results reported both for the liquid Merlin bulk and the liquid AD 169 strain, but no harmonization for (and by) the BAC preparation. Participants had used different matrixes for dilution (plasma, whole blood, urine) without an evident matrix-dependent difference in NAT harmonization being obvious from this study.

Commutability of CMV WHO IS in different matrices

The findings from the collaborative study for the WHO IS for HCMV described above did not concur with results obtained by a manufacturer of CMV NAT assays using the manufacturer’s specific protocols for testing human plasma or for testing whole blood. Dr Shiaolan Ho from Abbott described the matrix effect that was observed when the WHO IS for HCMV was diluted in whole blood compared with plasma and also described a commutability study with patient specimens to further evaluate the matrix effect. Patient specimens showed similar CMV-DNA quantitation regardless of diluent or extraction procedures used. The WHO IS for HCMV, on the other hand, was not commutable with patient specimens and exhibited a matrix effect when diluted in whole blood, which may complicate standardization across different quantitation methods and different laboratories.

The need for a commutable CMV reference material for viral load testing

Dr Angela Caliendo, representing the Pan American Society for Clinical Virology, presented the clinical perspective whereby non-harmonised CMV NAT assays revealed significantly different results for viral load values in patients which may potentially lead to different therapeutic decisions. Better assay harmonization would enable multicenter studies to be conducted to address key clinical questions. Evidence was presented demonstrating that a commutable calibrator may improve agreement in viral load values between assays, while a non-commutable calibrator does not improve agreement and may worsen agreement. Preliminary analysis of a study assessing the commutability of the HCMV IS using 8 CMV tests showed that the IS is commutable for the majority of the tests pairs. However, the interpretation of commutability studies assessing molecular assays is complex and complicated by high intra-assay variability, and may require modification of the data analysis approach used for chemistry tests.

SRM2366 as HCMV-DNA reference material with metrological traceability

Dr Mary Satterfield (NIST) summarized the standardization approach of the National Institute of Standards and Technology (NIST) which is based on certified reference materials (named as “Standard Reference Materials”, SRM), including synthetic reference materials where necessary. In the case of CMV NAT standardization, it is the material SRM 2366 which represents the complete genome of CMV Towne strain as artificial bacterial chromosome (BAC). It was stated that by
definition this material is a higher order reference material (value assignment in copies/ml) which is intended for value assignment of the number of amplifiable genome copies of CMV per volume, and might also play an important role in assuring consistency between different biological reference materials, e.g. the 1st WHO IS for HCMV and its replacements materials, when an appropriate reference method procedure is used.

**Commutability of WHO ISs for HCV-RNA**
Dr John Saldanha (Roche) provided an overview of the harmonisation efforts for measurements of HCV RNA using NAT assays. Commutability does not appear to be an issue with these standards, since different commercial HCV NAT tests are able to obtain similar results with clinical samples. This is likely to be because the materials used for preparing the standards closely resemble clinical test specimens. In contrast, issues such as the potency assignment of replacement standards and long term stability of these materials need further investigation. Dr Saldanha encouraged both the use of External Quality Assurance Schemes (EQAS) data to demonstrate commutability with a much larger cohort of samples than is possible in a WHO collaborative study and also the availability of well characterized clinical specimens for new NAT test validation.

**External Quality Assurance Schemes (EQAS) for molecular diagnostics**
Proficiency testing schemes, such as those organized by Quality Control for Molecular Diagnostics (QCMD), are increasing in both size and scope and participants frequently need these programs as part of the accreditation processes of the laboratories. For many markers it is obvious that the development of WHO ISs has led to the results reported in EQAS becoming much more consistent between the participating laboratories and Dr Bert Niesters (QCMD) presented examples of this for assays detecting HCV-RNA, HIV-1 RNA and HBV-DNA. As a result, WHO International standards and other international reference materials are strongly supported by EQAS organizers. The potential inclusion of these higher order reference materials into EQAS was discussed as a potential approach to evaluate their commutability. A pre-condition for such an approach is that the other samples used for comparison would need to fully represent clinical specimens.
Session 3: Reference Preparations for Serological assays

Prior to the presentation of individual case studies for reference preparations for both antibodies and antigens, Dr Phil Minor from NIBSC presented an overview of some of the common problems associated with the preparation of reference preparations for serological assays. These included the general problem of assay specificity where different assay methods might measure different targets (i.e. different measurands), which is an inherent problem with the complexity of serological assays (different virus clades, different antigens recognized and different biological assay principles). These issues were discussed in more detail in the presentations of individual case studies (briefly summarized below and in more detail in Appendix 2).

Reference Preparations for Antibodies

1st IS for Diphtheria Antitoxin Human
The measurement of anti-diphtheria antibodies is essential for evaluation of diphtheria vaccines in clinical trials, for population immunity studies and for the assessment of the immune status of individual patients. Dr. Paul Stickings (NIBSC) presented the details of a formal study, performed by NIBSC and ISS to investigate the commutability of the 1st IS for Diphtheria Antitoxin, which was undertaken as a follow up to the main collaborative study to value assign the standard but prior to the adoption of the standard by the WHO Expert Committee on Biological Standardisation (ECBS). Importantly, the study was conducted in coordination with an existing External Quality Assessment study and this was identified as a very convenient and efficient method for a commutability assessment of an International Standard. Although this study demonstrated a high degree of commutability of the 1st IS for Diphtheria Antitoxin Human, it was remarked that the scope and timing of the EQA before discussion of the IS with the ECBS was fortuitous, having depended on a special effort by an external organization to gather fresh clinical specimens.

Testing for Rubella IgG
Dr. Liliane Grangeot-Keros (Hôpital Antoine Béclère) presented the results of three recent EQA studies of clinical specimens from recent rubella vaccinees and persons with past infections which demonstrated variations of as much as ten-fold or greater in the determination of mean IU/mL across a broad range of currently used in vitro immunoassays. The underlying basis for currently observed inconsistency of in vitro assays for rubella IgG is thought to arise from the shift from “classic” use of whole virus antigens in indirect ELISA assays to “new generation” assays that utilize a variety of antigens (e.g., recombinant virus, recombinant proteins) and a variety of assay formats (e.g., sandwich, capture and competitive EIAs.) The resulting differences in measuring IgG levels can have very significant effects on clinical care (e.g., unnecessary revaccinations based on false negative tests and unnecessary elective abortions based on putative seroconversions in pregnancy) and population surveillance. A meeting with manufacturers is scheduled in September 2013 to determine whether individual manufacturers could establish independent assay-cutoffs

Hepatitis B Immunoglobulin Reference Preparations
Dr. Karl-Heinz Buchheit (EDQM) reported on the experiences of the EDQM with assay variation both in the context of the WHO study to establish the 2nd IS for Hepatitis B Immunoglobulin and in the context of a project to establish a cognate Biological Reference Preparation (BRP) as a secondary working standard. The results from these studies demonstrate that assigned values for
secondary or replacement reference preparations depend critically on the choice of kits used in the calibration study. In the absence of any systematic bias, conversion factors cannot be used to harmonize assay results since the assay values also depend on which reference preparation is used. Thus, potency measurements need to be validated independently for each immunoglobulin product, reference standard and test kit.

Reference Preparations for Antigens

Antigen markers
Dr Sigrid Nick (PEI) presented data from assays for HBsAg, HCV and HIV, which demonstrated that analytical sensitivity determined with standard preparations for these viral antigens correlated well with sensitivity for early detection of infection. However, pilot studies should be performed to investigate whether the treatment of standard materials during preparation, which may include lyophilization and inactivation of infectivity, alters antigen and epitope conformations, potentially affecting commutability with clinical samples. In addition, assay titrations of reference materials can be strongly affected by the dilution matrix. Therefore, recommendations should be made for use of suitable dilution matrices in the context of international collaborative studies and other use settings. The nature of the reference material, and especially the expression system for a recombinant material, can create significant bias in assay calibration. Naturally derived materials appear to be superior to recombinant materials. Due to the considerable variation among certain viral pathogens (types, subtypes, genotypes and mutants), the materials selected for preparation of International Standards should represent the most prevalent variants in order to be widely applicable. Molecular characterization of the antigen source, including genotyping and sequencing should be performed if possible and use of antigens that express genetic mutations should be avoided.

Comparative Assay Performance Obtained From a Large EQA Serology Scheme
Through 46 EQA schemes, the UK NEQAS obtained data on 12 serological and 9 NAT assays from approximately 1500 laboratory participants. Nine WHO IS were included in the various schemes. In defining commutability of the IS, attention was drawn to the clinical importance for agreement of qualitative assays at the cut-off (to avoid false determinations related to immune status) and for agreement of quantitative assays in IU in order to assure consistency with medical guidelines such as for vaccination/booster dosing and post exposure prophylaxis. Dr Brigitte Senechal, (UK NEQAS Microbiology) provided some exemplar data and reported disagreement in the measured titers of EQA specimens across anti-HBs and Rubella IgG assays, which raised questions about the assay agreement for the reference preparations, whether vaccinee serum behaves differently than serum from naturally infected persons, whether assay differences could be due to variability in host responses to the same or different virus strains, and whether the apparent lack of assay agreement could be attributed to differences in assay designs.
Session 4: General Discussion

Participants at the meeting recognized the challenges in harmonizing assays for the in vitro detection of infectious markers. Much of the preliminary discussion was focused on the general observations that were captured from the individual case studies that had been presented. These included issues of assay specificity (different assay methods might measure different targets), complexity of the target measureands (antigenic differences, different viral clades, different antibody compositions), and the complexity of serological assay design generally. Building on this discussion and expanding on some of the principles raised, there then followed a more focused discussion on the assessment of the commutability of WHO biological reference preparations for the in vitro detection of infectious markers.

In assessing the commutability of a reference preparation, a number of central issues arise. First is the consideration of what clinical use will be made of the test results. For instance, incommensurable assays may be used appropriately either to monitor responses in vaccine trials or to determine the immune status of individual patients. A second issue is that the apparent non-commutability of reference preparations may in fact reflect fundamental differences in assay characteristics affecting their measurands. This arises because, for instance, serologic assays vary considerably in their design (e.g., nature of the binding materials and diverse assay formats etc.). Additionally, the targets of the assays are themselves complex including biologic variability of the analytes (e.g. antibody mixtures reflecting different host responses, antigenic differences reflecting variable genotypes and genetic mutants). A third consideration is matrix effects. These are seen commonly and are a true issue of non-commutability when there is commonality of the measurand across assays. A better understanding of the measurands would help to assess whether the issue of non-comparable assay results is due to non-commutability of the reference preparation.

The value of local testing of large numbers of clinical samples to validate the commutability of a reference preparation in a small number of assays was raised. It was pointed out that the best time for such an exercise is when the laboratory changes its assays. However, clinical laboratories generally will lack access to high titer specimens for such testing, limiting the value of the assessment. Furthermore, pairwise comparison of assays cannot be used to generalize commutability across assays that are not directly compared. If a reference preparation is shown commutable for assays A and B with one set of samples and separately shown commutable for assays B and C with a different set of samples, one cannot presume commutability of the reference preparation for assays A and C. Nevertheless, commutability of a reference preparation in a large number of pairwise assay comparisons using different clinical samples is informative to some degree. Alternatively, it was suggested that lot release laboratories would be in a better position to test multiple assays with common sets of clinical samples.

A question was raised whether there is feasibility and utility to quantitate the degree of commutability of a reference preparation. The ‘yes’ or ‘no’ approach currently employed in the literature does not give a guide to how effective a reference preparation may be in reducing intra-method or intra-laboratory variability, and preparations that are ‘non-commutable’ by a strict definition may still be of value. An example of one approach for a multi-method study had been shown in Dr Caliendo’s presentation of a CMV commutability study. However, a basic uncertainty exists how to take this forward from a statistical standpoint, and the best approach that could provide a quantitative measure that would reliably predict the value of a reference preparation to reduce the
variability between laboratory assays. Given the limitations in determining commutability, the suggestion was made that the instructions for use of WHO reference preparations should provide more detailed information about what is known as well as instructions for validation with specific assays. The possibility for use of correction factors (or correction functions) with some assays should not be overlooked if correlations are established with large studies.

A general observation was made that specificity of the measurand and the corresponding ability to harmonize assays is greatest for NAT, can be comparable for some antigen assays, and is least for antibody assays. The variability in the results reported using different biological assay methods leads to the concept that care providers, as the end users, need better education on the variability of these assays, especially for serology, and the implications for assessments of immunity. However, it is unclear who bears this responsibility (i.e., WHO, assay manufacturers, medical societies.) Although the results of EQA assessments are public and make this clear, the care providers are generally unaware of such data and depend instead on recommendations of medical experts. Considering this situation, a suggestion was made that a message from WHO to national organizations would be valuable in this regard, for instance as advice to vaccine policy setting organizations. As an example, the recent UK message on protective levels of anti-HBs (namely that >10 mIU/mL is protective, but a level of >100 mIU/mL is better) demonstrate that messaging can help physicians to address assay uncertainties. Reference preparations also can be assigned different units for use in different assays. This might be considered for measles antibodies where different IU could be assigned for neutralization versus binding.

A WHO representative committed to bringing the concept of an educational effort on variability in the results reported using different biological assay methods forward to the vaccines department. In this context, mention was made of the fact that WHO already posts on its website the results of the international collaborative studies of reference preparations. But, there is presently no database that aggregates worldwide experience with assay comparisons using reference preparations. For example, the commutability of the IS for rubella IgG and anti-HBs were studied in Canada in the context of the development of secondary standards and large variations in assay performance were found. Such information is not widely shared, though it was noted that the AACC is now trying to do this. It is important that negative as well as positive study results should be reported. This might be consistent with editorial policies, for example that of the “Clinical Chemistry” journal, where the goal is to publish studies that would move the field forward. Emphasis was stressed on prioritization of feedback for assays with high clinical impact.
General consensus of the meeting

Based on the presentations and discussions heard at this consultation, the collaborating center representatives drafted the following consensus statements:

- Pilot studies should be performed to investigate the effects of processing (e.g. lyophilization, inactivation of infectivity, suitability of dilution matrices) on candidate international reference preparations prior to international collaborative studies.
  - This should be done in multiple laboratories and should include multiple assays.
- As a minimum, a small number of clinical samples should be included routinely in the international collaborative studies of international reference preparations.
  - Ideally, these samples should be chosen to represent the diversity and range of reactivities seen in the clinical conditions associated with the measurand.
  - Pooling of clinical samples may be required for validation of assays to avoid the more complex logistics of handling large numbers of such samples.
- Formal studies of commutability of international reference preparations should be undertaken when technically and logistically feasible, with subsequent decisions made as appropriate on the suitability for use and/or labeling of the reference preparation.”
- Where feasible, EQA studies that include clinical samples should be leveraged to include international reference materials as a basis to assess their commutability.
- Written information accompanying international reference materials should be enhanced for specificity with respect to their intended use, the nature of the measurand as it exists in the material, the scope of demonstration of their commutability, identification of suitable matrices for dilution, and directions for local validation of the commutability of assays with the reference preparation, including the use of a correction factor where feasible to correct a systematic bias of an assay.
- Cooperation is needed to improve the availability of clinical samples for use in validation of international reference preparations.
  - WHO may wish to consider development of a statement on the ethics for research use of de-identified surplus clinical specimens
- WHO and the collaborating centers should establish a co-operative effort with the AACC initiative for the harmonization of clinical laboratory test results which emphasizes the importance of commutability of reference materials.
- Funding opportunities for conducting commutability studies should be sought among stakeholders e.g. academia, device manufacturers and government.
References


Appendix 1

Reference preparations for NAT assays – Presentation of case studies

Presentation: 1st WHO IS for Human Cytomegalovirus (HCMV) for Nucleic Acid Amplification (NAT)-Based Assays and its commutability

Dr Jacqueline Fryer (NIBSC)
Dr Fryer described the standardization project performed by NIBSC in close cooperation with the community of clinical virologists. Patient specimens of appropriate volume and with sufficient CMV-DNA concentration were not available for production of the WHO IS; therefore a cell culture system was chosen, and the final WHO IS candidate preparation (Merlin strain, lyophilized) was formulated with universal buffer to be spiked into different patient matrixes (plasma, whole blood, CSF, urine etc). The collaborative study included the liquid counterpart of the WHO IS candidate, a liquid cell culture derived AD169 strain and a bacterial artificial chromosome (BAC) with the complete viral genome of the Merlin strain. The collaborative study showed pronounced harmonization of assays by the WHO IS candidate with regard to results reported both for the liquid Merlin bulk and the liquid AD 169 strain, but no harmonization for (and by) the BAC preparation. Participants had used different matrixes for dilution (plasma, whole blood, urine) without an evident matrix-dependent difference in NAT harmonization being obvious from this study.

Presentation: Commutability of CMV WHO IS in different matrices

Dr Shiaolan Ho (Abbott)
The findings from the collaborative study for the WHO IS for HCMV did not concur with results obtained by a manufacturer of CMV NAT assays using the manufacturer’s specific protocols for testing human plasma or for testing whole blood. Dr Shiaolan Ho from Abbott described the matrix effect that was observed when the WHO IS for HCMV was diluted in whole blood compared with plasma. A commutability study was subsequently conducted following guidance (EP14-A2) of the Clinical and Laboratory Standards Institute (CLSI) to further evaluate the matrix effect. The WHO IS for HCMV was tested against 20 positive patient plasma specimens, which were used as the standard of comparison. Patient specimens were diluted in plasma and whole blood, and tested along with the IS. Plasma procedure (e.g. extraction) was used for testing specimen and the IS diluted in plasma matrix, whereas whole blood procedure was used for testing dilutions made in both plasma and whole blood matrices. Regression analysis was performed using the means of patient specimens, and two-tailed 95% prediction interval was calculated. Results for the WHO IS for HCMV were evaluated relative to the prediction interval. Patient specimens showed similar CMV-DNA quantitation regardless of diluent or extraction procedures used. The WHO IS for HCMV, on the other hand, was not commutable with patient specimens and exhibited a matrix effect when diluted in whole blood. This matrix effect appeared to “reduce” the apparent DNA quantitation of the IS by approximately 0.4 log, or about 2.5 fold. This reduction introduces an analytical and a calibration bias. If an assay is standardized to the WHO IS for HCMV diluted in whole blood, since the IS runs lower, samples will be reported higher in measured concentration in IU. The matrix effect in whole blood and the potential bias could complicate standardization across different quantitation methods and different laboratories. To eliminate the bias and to achieve consistent standardization, mitigation is necessary. For this case, it was proposed by Dr. Ho that dilution of the WHO IS in plasma dealt with the problem, since the WHO IS for HCMV diluted in plasma performed similarly to patient
specimens regardless of extraction procedure. Given the commutability issue identified with the WHO IS for HCMV, Dr. Ho suggested that a revision to the instructions of use should be considered. The revision should caution users on potential impact from diluent matrix and to suggest characterization in order to define an “appropriate” matrix. For laboratories in routine testing of whole blood specimens, plasma could be an appropriate matrix for dilution of the WHO IS for HCMV.

**Presentation: The need for a commutable CMV reference material for viral load testing**

Dr Angela Caliendo (Pan American Society for Clinical Virology)

Dr Caliendo presented the clinical perspective whereby non-harmonised CMV NAT assays revealed significantly different results for viral load values in patients which may potentially lead to different therapeutic decisions. These include important clinical decisions such as should treatment be initiated, is it time to stop therapy, is the patient failing therapy, and what viral load predicts disease? Better assay harmonization would enable multicenter studies to be conducted to address these key clinical questions. An important component of this harmonization is calibration of tests to the international standard; this must be done by manufacturers and individual laboratories (using laboratory developed tests). One of the principal characteristics of this International Standard is that it should be commutable with clinical samples. A study of cytomegalovirus (CMV) viral load assays showed that using a commutable calibrator improved the agreement in viral load values between two tests, while using a non-commutable calibrator did not improve agreement. When this work was expanded to study five different assays; results again showed that a commutable calibrator may improve agreement between viral load values and it did not worsen agreement. While a non-commutable calibrator does not improve agreement and it may actually be detrimental. A similar commutability study was performed with the WHO IS for HCMV and 50 clinical samples measured using 8 assays. Although this study has not been fully evaluated yet, there was better agreement of viral load values when results were reported as international units/ml (IU/ml) compared to earlier studies that reported results as copies/ml. In addition, preliminary results show that overall the WHO IS for HCMV is commutable with the majority of the assay pairs, but there are tests for which the WHO IS for HCMV is not commutable. The point was made that, given the complexity of assessing commutability, for molecular tests where assay variability is generally greater than that seen with chemistry tests (for example glucose and cholesterol), there may need to be some flexibility in data analysis so that the degree of commutability can be assessed. Moreover, commutability studies of molecular standards are difficult to conduct in part because of the difficulty obtaining large volumes of clinical samples. Digital PCR was also discussed as a new technology for calibrator-independent quantification of clinical samples as well as a potential tool for characterization of reference materials and for replacement studies. In digital PCR amplification reactions are performed in numerous partitions simultaneously allowing calculation of the analyte concentration based on its Poisson distribution.

**Presentation: SRM2366 as HCMV-DNA reference material with metrological traceability**

Dr Mary Satterfield (NIST)

Dr Satterfield summarized the standardization approach of the National Institute of Standards and Technology (NIST) which is based on certified reference materials (named as “Standard Reference Materials”, SRM), including synthetic reference materials where necessary. In the case of CMV NAT standardization, it is the material SRM 2366 which represents the complete genome of CMV.
Towne strain as artificial bacterial chromosome (BAC). The sequence of SRM 2366 was confirmed in different regions used as targets by amplification systems. The material was quantified by different digital PCRs using six different target regions. Results were consistent, only an amplicon of (unusually) large size gave slightly lower results. Stability of the material was shown for temperatures up to 37°C. It was included in a QCMD study with 233 participants in 35 countries. SRM 2366 is a post-extraction material, and in the QCMD study was analysed by direct spiking into the amplification reaction. The distribution of the QCMD results was approximately Gaussian, peaking in the range of 10⁶ copies/ml which overlaps well with the value determined by NIST. However, there were also few laboratories providing much lower or higher results. It was stated that by definition this material is a higher order reference material (value assignment in copies/ml) with metrological traceability. It is intended for value assignment of the number of amplifiable genome copies of CMV per volume, and might also play an important role in assuring consistency between different biological reference materials, e.g. the 1st WHO IS for HCMV and its replacements materials, when an appropriate reference method procedure is used. In the discussion the point was raised if traceability should/may rely on digital PCR and to which extent further physical methods (OD, phosphate measurement) might contribute.

Presentation: Commutability of WHO ISs for HCV-RNA

Dr John Saldanha (Roche)

Dr Saldanha provided an overview of the harmonisation efforts for measurements of HCV RNA using NAT assays. The first WHO International Standard for HCV RNA for NAT tests was established in the mid-1990s in order to harmonize results between NAT tests (mainly in-house tests) which were being used for testing plasma for further manufacture. The first and second HCV International Standards consisted of a clinical plasma sample, positive for HCV RNA and anti-HCV, diluted in pooled, negative human cryosupernatant. The first WHO International Standard for HCV RNA was widely adopted and was successful in harmonizing in-house and commercial NAT tests. Commutability did not appear to be an issue with this standard, since different commercial HCV NAT tests were able to obtain similar results with clinical samples. Where issues were reported, such as under quantitation of specific HCV genotypes, further investigations showed that these were due to sub-optimum test performance arising from primer and/or probe mismatches with certain HCV genotypes rather than lack of commutability of the International Standard with clinical specimens. However, such reports were relatively rare since almost all commercial in-house and commercial NAT tests amplify the conserved 5’ non-coding region of the HCV genome (although polymorphisms have also been reported in this region of the HCV genome). Although commutability does not appear to be an issue with the WHO International Standards for HCV RNA NAT tests, the assignment of potency to replacement HCV International Standards can be challenging. The accuracy and precision of NAT tests for HCV, especially quantitative tests, are continually improving so that any ‘drift’ in the International Unit from one WHO International Standard to the next replacement will be problematic. Such drift is possible given that the establishment of a replacement standard is obtained through a consensus process involving as many different tests as possible. A second issue, which has become apparent with the third and fourth International Standards for HCV RNA, is the long-term stability of the standards. Limited studies have indicated that long term stability may be related to several different issues such as sample type, dilution matrix, lyophilization and inactivation of the standard. In conclusion, commutability does not appear to be a major issue with the WHO International Standards for HCV RNA NAT tests, probably because the materials used for preparing the standards closely resemble clinical test
specimens. However, issues such as the potency assignment of replacement standards and long term stability need further investigation. The use of External Quality Assurance Schemes (EQAS) data to demonstrate commutability with a much larger cohort of samples than is possible in a WHO collaborative study and the availability of well characterized clinical specimens for new NAT test validation should be encouraged.

**Presentation: External Quality Assurance Schemes (EQAS) for molecular diagnostics**

Dr Bert Niesters (QCMD)

Proficiency testing schemes, such as those organized by Quality Control for Molecular Diagnostics (QCMD), are experiencing both an increase in the number of participating molecular diagnostics laboratories, and also in the number of different assays in use, reflecting the growing importance of these measurements. Participants frequently need these programs as part of the accreditation processes of the laboratories. In addition, among molecular assays for infectious markers, there has been an increase in the use of quantitative instead of qualitative assays over the past few years. For many markers it is obvious from EQAS results that the range of reported results, for quantitative assays, may be very wide, especially before international standardization or harmonization is introduced. This wide range of results between assays makes general guidelines for interpretation of test results and for triggering decisions on therapy of patients impossible. However, over the past decade, the development of WHO ISs alongside the increasing availability and use of commercial assays, has led to the results reported in EQAS becoming much more consistent between the participating laboratories and Dr Niesters presented examples of this for assays detecting HCV-RNA, HIV-1 RNA and HBV-DNA. As a result, WHO International standards and other international reference materials are strongly supported by EQAS organizers. Participants in EQAS are encouraged to report in International Units as soon as a WHO IS has been made available. The observation was made that International Units were easily accepted by the laboratory community for HCV-RNA and also increasingly for HBV-DNA. However, for HIV-1 RNA many physicians prefer to stick with copies/ml, despite a WHO IS available assigned with IU/ml. The range of available calibrators was discussed, extending from NIST and WHO materials to commercial control materials and internal reference materials (IRM) used by IVD manufacturers. The IRM should be characterized with regard to strain and sequence, its stability and homogeneity should be known, and where possible, the IRM is calibrated and traceable to higher order reference materials or International Standards if available. Specimens included in EQAS should be also be characterized using these reference materials. The potential inclusion of higher order reference materials into EQAS was discussed as a potential approach to evaluate their commutability. A pre-condition for such an approach is that the other samples used for comparison would need to fully represent clinical specimens.
Appendix 2

Reference preparations for Serological assays – Presentation of case studies

Reference Preparations for Antibodies

Presentation: Commutability issues in serological references

Dr Philip Minor (NIBSC)
3rd IS for Anti-Measles (Plasma)

The findings of a second collaborative study (WHO/BS.07/2076) to establish potency of the 3rd IS for Anti-Measles (Plasma) relative to the 2nd IS and 1st IS were described. Attention was drawn to divergence of potency measurements by several ELISA methods (Enzygnost, Serion and IBL) compared with the agreement in results from PRNT and the Enzygnost ELISA. These differences precluded establishment of unitage for ELISA methods despite calibration against the IS. Additionally, the Enzygnost ELISA produced higher (approximately two fold) estimates of potency than PRNT or other methods used in the past (e.g. HI). The findings were taken to illustrate a general problem wherein different assay methods might measure different things (i.e. different measurands.) This phenomenon is often interpreted as non-commutability of the reference preparation for different assays. However, the observation of non-commutability may relate to fundamental differences in the assay methods including their measurands. In this case ELISA and PRNT appear to measure different things, and clinical correlation for protective human immunity exists only for potency when PRNT is applied. The possibility exists that international unitage for ELISA could be established to demonstrate the presence of a vaccine response, but not establish whether the response was protective.

Proposed 1st IS for Influenza H5N1 antibodies and 1st IS for Influenza H1N1 antibodies

A collaborative study (WHO/BS/08.2085) was undertaken to validate a proposed 1st IS for human H5N1 antibodies to enable the efficacy comparison of pandemic vaccines. The candidate IS (07/150) was made from vaccinee plasma raised against a clade-1 H5N1 (NIBRG14) derived vaccine. A candidate sheep antiserum to clade-1 H5N1 was also considered to determine whether it would be possible to use sheep antiserum to standardize human serology to save time in a pandemic outbreak. A comparison was made of assay results for virus neutralization (VN) and hemagglutination inhibition (HI) using assays based on antigens from two clade-2 and one clade-1 strains of H5N1. The results demonstrated that candidate IS 07/150 reduced by 50% the inter-laboratory variability of VN and HI assays for antibody induced by vaccination with the same clade-1 derived virus when the assays were based on antigens also from the same clade-1 virus. However, calibration against 07/150 did not reduce the variability of antibody assays when the assay antigens were derived from clade-2 viruses. Sheep serum against clade-1 virus somewhat reduced variability of HI assays, but not VN assays.

In a similar fashion, a collaborative study was undertaken to establish a 1st IS for antibodies to pandemic influenza H1N1. Candidate IS 09/194 (a lyophilized preparation) reduced by 50% the inter-laboratory variability of HI and VN assays for antibodies against H1N1 in both post-vaccine and convalescent sera. However, the relationship between the HI and VN assays varied between laboratories.
A general conclusion was drawn that no serum sample can behave the same way as all others in all assays. Thus, commutability of a reference serum depends on the assays involved (e.g., ELISA, PRNT, HI, etc.), the serum itself (e.g., sheep versus human, standard versus test), and the antigens presented in the assay (e.g., virus clades, antigens recognized by different sera, biologic details). Nevertheless, international reference preparations can have a major effect for improving agreement among results of different assays. This was shown for assays of antisera to H5N1 and H1N1 when the antigens in the assays came from the same virus as was used to make the vaccine strain and when the predominant natural infection was expected to be due to the same clade of virus.

**Presentation: 1st IS for Diphtheria Antitoxin Human**

Dr Paul Stickings (NIBSC)

The level of anti-diphtheria antibodies in serum is measured in International Units (IU) and forms the basis of immunological correlates of protection for diphtheria vaccines. The measurement of anti-diphtheria antibodies is essential for evaluation of diphtheria vaccines in clinical trials, for population immunity studies and for the assessment of the immune status of individual patients. Most routine analysis of human sera is done by immunoassay for which a species specific reference preparation is required. These assays include “in-house” immunoassays as well as commercial diagnostic ELISA kits. Dr. Stickings presented the details of a formal study, performed by NIBSC and ISS to investigate the commutability of the 1st IS for Diphtheria Antitoxin, 10/262 (a normal human immunoglobulin preparation). This study was undertaken as a follow up to the main collaborative study to value assign 10/262, but prior to the adoption of the standard by ECBS, and was conducted in coordination with an existing External Quality Assessment study. A total of 16 laboratories tested a panel of 150 samples, which included 148 human serum samples together with the new IS (10/262) and a human serum working standard (00/496). Methods used included toxin neutralization tests, in-house immunoassays and commercial ELISA kits. Results from 14 laboratories were used for the commutability assessment which was done using pairwise comparisons of all laboratory pairs by linear regression analysis, with normalised residuals for the new IS used to determine comparisons for which it was commutable or non-commutable. The results suggest that the new IS (10/262) is commutable with human serum samples, showing comparable behaviour to native human serum samples in 96% of 182 method pair comparisons. The samples of the working standard 00/496 were commutable in 93% of method pair comparisons. These data were also assessed using a multivariate approach, principal components analysis, with the results here also suggesting commutability of the new IS. These results supported a statement in the indications for use of the IS that, “The results of an international collaborative study suggest that this standard is suitable for use as a reference preparation in toxin neutralization tests and in vitro immunoassays (including ELISA), and that the standard showed comparable behavior to native human samples in the majority of different assay methods used.”

This study demonstrated a high degree of commutability of the 1st IS for Diphtheria Antitoxin Human in a timely and rigorous investigation of assays. However, it was remarked that the scope and timing of the EQA before discussion of the IS with the ECBS was fortuitous, having depended on a special effort by an external organization to gather fresh clinical specimens and not a routine EQA survey. Also, it was noted that the study could have been improved by prequalification of the laboratories (for assay proficiency), optimization of the range of reactivity of the panel specimens and incorporation of dilutions of the candidate reference materials. Occasional disparities in the
apparent commutability of the reference materials when analyzed in reverse ordered pair-wise comparisons of the same assays was attributed to an artifact of the statistical method used (least squares rather than orthogonal regression) and the effect of borderline values. A suggestion also was made to consider plotting relative residuals over the range of clinical reactivity of the clinical specimens in order to examine the possibility of bias at lower levels, where determinations of antibody potency are highly clinically relevant (e.g. to determine the need for booster vaccination of individuals and to survey immunity in a population).

**Presentation: Testing for Rubella IgG**

Dr. Liliane Grangeot-Keros (Hôpital Antoine Béclère)

Dr. Grangeot-Keros, presented the results of three recent EQA studies of clinical specimens from recent rubella vaccinees and persons with past infections which demonstrated variations of as much as ten-fold or greater in the determination of mean IU/mL across a broad range of currently used in vitro immunoassays. In particular, the quantitative results obtained with the widely used Abbott Architect and the Roche Cobas 6000/Elecsys/Modular assays showed significant discrepancy despite kit calibration against the 3rd WHO reference standard, RUBI-1-94. Furthermore, EQA studies in 139 laboratories of human blood specimens prepared at the clinical “cut-off” of 10 IU/mL (associated historically with immunity when tested in an HI assay) showed highly variable results with overall findings of ca. 10% false negativity and 28% equivocal results across laboratories. Elevated and disparate rates of serologically determined rubella infection found in a 2012 UK study of diseases in pregnancy also suggested inaccuracy in the serial determinations of antibody levels by various assays. An EQA study of antibody levels in recent vaccinees showed up to five-fold variation in the determination of mean IU/mL. A comparison of the studies indicated decreased sensitivity of the Roche Elecsys assay compared with other assays for rubella IgG potency determination after recent immunization, but, conversely, higher sensitivity for potency determinations at later times. A suggestion was made that Western immunoblots might provide a reference method to resolve some assay discrepancies. As an example, resolution testing with a Western blot (Mikrogen immunoblot) showed that 10 low reactive samples with positive results on the Roche Elecsys assay and negative results on the Beckman Access/DXi assay were true positive, demonstrating a difference in assay sensitivities. This was evident despite the stated assay cutoffs of 15 IU/mL for the Access assay versus 10 IU/mL for the Roche assay. The underlying basis for currently observed inconsistency of in vitro assays for rubella IgG is thought to arise from the shift from “classic” use of whole virus antigens in indirect ELISA assays to “new generation” assays that utilize a variety of antigens (e.g., recombinant virus, recombinant proteins) and a variety of assay formats (e.g., sandwich, capture and competitive EIAs.) The resulting differences in measuring IgG levels can have very significant effects on clinical care (e.g., unnecessary revaccinations based on false negative tests and unnecessary elective abortions based on putative seroconversions in pregnancy) and population surveillance. An example was given of a woman with a subsequently well documented vaccination history who received a serological diagnosis of rubella infection during pregnancy based on assays performed serially, but in two different laboratories. An additional observation is that a cut-off of 10 IU/mL to define rubella immunity using current assays suggests incorrectly that up to 5% of previously immunized pregnant women may require revaccination. Cross sectional studies suggest that a cut-off of <4 IU/mL might be more accurate to determine susceptibility to rubella infection. However, the consistency of current assays to define either a qualitative or a quantitative result for rubella IgG in IU is questionable. These issues have been under discussion at a series of meetings since February 2012 on “Progress towards Rubella
elimination and CRS (congenital rubella syndrome) prevention in Europe.” A meeting with manufacturers is scheduled in September 2013 to determine whether individual manufacturers could establish independent assay-cutoffs based on their assessment of assay specificity using a well-defined panel of Western blot/immunoblot negative samples and qualitative assay sensitivity correlated with detection of immunity.

Comments were heard that the cut-offs for rubella IgG assays need to be focused not on antibody level detection per se, but on a determination of an immune state. In essence, a single vaccination confers lifelong immunity, which is reflected in the detection of even a very low level of antibody. It was noted that IgG levels of 5-10 IU/mL by HI have been correlated with presence of cell mediated immunity and with secondary immune responses to vaccination. In contrast, levels of 0-5 IU/mL were correlated with primary immune responses indicative of prior susceptibility to infection. The difficulty that has arisen concerns the lack of correlation of IU as measured by current non-HI methods with the actual immune status. Presumptive denomination of current assay results in IU has therefore only confused the clinical picture. Due to the above mentioned discrepancies and their consequences, the suggestion was made that consecutive samples must always be tested in the same laboratory, with same assay and in the same run.

**Presentation: Hepatitis B Immunoglobulin Reference Preparations**

**Dr Karl-Heinz Buchheit (EDQM)**

Dr. Buchheit reported on the experiences of the EDQM with assay variation both in the context of the WHO study to establish the 2nd IS for Hepatitis B Immunoglobulin and in the context of a project to establish a cognate Biological Reference Preparation (BRP) as a secondary working standard. First it was noted that results of 19 assays studied in 34 laboratories revealed a 20 percent variation in the geometric mean potency of the 2nd IS when calibrated against the 1st IS. Calibration of the EDQM BRP for Hepatitis B Immunoglobulin demonstrated a biphasic distribution of results among six kits used in 20 laboratories, indicating that different kits are measuring different things. A potency assignment of 55 IU/mL was made for the BRP based on the overall mean in the face of the observed measured range of 93-108% of the mean. Ranking of the potency of six commercial Hepatitis B Immunoglobulin products was not conserved based on calibration with the 1st IS, the 2nd IS or the BRP. The results were taken to demonstrate that assigned values for secondary or replacement reference preparations depend critically on the choice of kits used in the calibration study. In the absence of any systematic bias, conversion factors cannot be used to harmonize assay results since the assay values also depend on which reference preparation is used. Thus, potency measurements need to be validated independently for each immunoglobulin product, reference standard and test kit.

It was noted that the absolute differences in mean potency of the BRP and 2nd IS as determined with the studied test kits was not large. Additionally, a variation of 20% among test kits of the type available was to be expected. Nevertheless, such variation in the potency measurement of Hepatitis B Immunoglobulin products is problematic. For this reason, the Japanese control authority now uses only a single assay for potency assessments of Hepatitis B Immunoglobulin products.
Reference Preparations for Antigens

Presentation: Antigen markers

Dr Sigrid Nick (PEI)

HBsAg
Analytic sensitivity of commercial HBsAg assays with a variety of monoclonal and polyclonal methods for antigen capture and detection is determined at the PEI by testing against an in-house standard that has been calibrated against the 2\textsuperscript{nd} IS for HBsAg. It was noted that the Enzygnost test is among the most sensitive tests for HBsAg even though this test uses a polyclonal antibody capture rather than a monoclonal capture reagent. Dr Nick explained that, at the PEI, the relative clinical sensitivity of various HBsAg assays is determined using a scoring system based on time to detection of positivity in 30 seroconversion panels. Sensitivity scores for commercial assays (reflecting an overall two week difference in time to first detection among kits) generally correlated with their analytical sensitivity as calibrated against the PEI standard. However, a significant matrix effect resulting in an underestimate of test sensitivity was seen when the PEI standard was diluted in fetal calf serum versus base matrix of human serum. Batch testing with dilutions of the PEI standard has been highly successful in detecting drift of assay performance, including an instance where an unreported manufacturing change was detected. Investigations have shown large differences in the sensitivity of commercial test kits to detect HBsAg of HBV genotype/subtypes D/ayw3, E/ayw4, F/adw4, and S gene and other mutant viruses. A significant difference in the analytical sensitivity of commercial test kits also has been shown using cut-off determination with the WHO 2\textsuperscript{nd} IS 00/588.

Hepatitis C core Antigen
Assays for Hepatitis C core Antigen are used both for early detection of HCV infection and for therapy monitoring. There are two major antigens p23 and p21, and one minor antigen p16. There are assays both for individual detection of HCV core Antigen and for detection of the antigen in combination with HCV antibodies. Assay sensitivity is generally independent of virus genotype, but can vary. One study (Ross et al. 2010) showed that quantitation of HCV antigen by the Abbott Architect assay was not much affected by genotype, but there was an exception for genotype 2. Another study (Tokida et al. 2000) demonstrated that L23R and T49P mutations in the HCV core Antigen affect its detection by the Immucheck F-HCV Ag Core assay. The relative sensitivity of commercial assays for detection of HCV infection was compared by the PEI using panels of seroconversion sera. Disparities in first time of detection of up to 28 days were observed. Titration of a candidate lyophilized standard for HCV core Antigen showed that combination antibody/antigen tests were less sensitive for detection of antigen than tests for antigen alone, with as much as a thousand fold difference in end-point titer. A calibration study for the standard would need to take into account this marked assay variation.

HIV p24 Antigen
Genetic variation of HIV is well recognized with the existence of virus types, clades and recombinant forms. Marked virus diversity is a hallmark both of infections in individuals and populations. Nevertheless, HIV p24 shows significant homology both within single patients (97.1-99.1%) and between patients (91.5-93.8%). Assays to detect HIV antigen either alone or in combination with HIV antibodies are used in diagnosis of HIV. The 1\textsuperscript{st} international reference
material for HIV antigen (NIBSC90/636) is a peptide isolated from a detergent treated virus preparation which has an assigned potency of 1,000 AU/mL. A recombinant HIV p24 protein prepared by Roche Corporation is used by the PEI. For unexplained reasons, the latter material is not suitable for use in calibration of the Abbott Prism assay. Bio-Rad distributes an AFSSAPS developed HIV antigen reference material which consists of inactivated HIV-1 virus that was pelleted from HIV-1\textsubscript{Brn} cultures and diluted in human serum. In the EU, tests for HIV antigen are CE marked only if they can detect <2 AU/mL of the international reference material. When the limit of detection of HIV antigen exceeds 2 AU/mL, detection of HIV p24 antigen does not enhance the sensitivity of combination antibody/antigen tests for early diagnosis of HIV infection.

General observations regarding tests for these viral antigens included the following:

- For assays that detect HBsAg, HCV and HIV antigens, analytical sensitivity determined with standard preparations correlated well with sensitivity for early detection of infection.
- Treatment of standard materials during preparation, such as lyophilization and inactivation of infectivity, may alter antigen and epitope conformations affecting commutability. Pilot studies should be performed to investigate this.
- Assay titrations of reference materials can be strongly affected by the dilution matrix. Therefore, recommendations should be made for use of suitable dilution matrices in the context of international collaborative studies and other use settings. Investigation of the commutability of reference preparations for antigens should incorporate testing of high, medium and low reactive native specimens (e.g. seroconversion samples.)
- The nature of the reference material, and especially the expression system for a recombinant material, can create significant bias in assay calibration. Naturally derived materials appear to be superior to recombinant materials.
- Due to the considerable variation among certain viral pathogens (types, subtypes, genotypes and mutants) the materials selected for preparation of International Standards should represent the most prevalent variants in order to be widely applicable. Molecular characterization of the antigen source, including genotyping and sequencing should be performed if possible and use of antigens that express genetic mutations should be avoided.
- Lack of assay precision for serological tests is not evidence of lack of commutability. Assay variations (CVs) at the cut-off of antigen detection are typically up to 10% for instruments and 15% for ELISA tests. Inter-lot variations in the range of 30% are expected and acceptable.

Additionally, it was noted that combination antibody/antigen detection assays can be falsely negative due to the presence of immune complexes when there is a high viral load. NAT assays may be preferred for this reason, or a combination of tests.

**Presentation: Comparative Assay Performance obtained from a Large EQA Serology Scheme**

Dr Brigitte Senechal, UK NEQAS Microbiology

Through 46 EQA schemes, the UK NEQAS obtained data on 12 serological and 9 NAT assays from approximately 1500 laboratory participants. Nine WHO IS were included in the various schemes. Quantitative results were reported in IU only for some serological assays, i.e., HBsAg (1 assay), anti-varicella (4 assays), anti-HBs (10 assays) and anti-rubella (12 assays). In defining commutability of the IS, attention was drawn to the clinical importance for agreement of qualitative
assays at the cut-off (to avoid false determinations related to immune status) and for agreement of quantitative assays in IU in order to assure consistency with medical guidelines such as for vaccination/booster dosing and post exposure prophylaxis. The following observations were made:

**HBsAg**

For HBsAg, the UK requires that a screening assay should be able to detect 0.05 IU/mL. To investigate assay proficiency at the cut-off, surveys were done with an EQA serum specimen containing 0.10 IU/mL of HBsAg (genotype A, adw2). All 10 assays in the study yielded positive results in nearly all laboratories; however, a small number of false negative results were obtained. Further studies are under consideration using lower level test specimens and specimens of different genotypes.

**VZV IgG**

For four quantitative assays of VZV IgG, analysis was performed on titers of four specimens which spanned a range of 100 to 1300 mIU/mL (defined as the average of the assay medians) and the question was explored whether titers were comparable in the linear range of the assays and whether cut-offs were consistent. There were 50 sets of results for each EQA specimen. Across the assays, linearity was maintained for specimens containing from 100 to 491 mIU/mL of VZV IgG. There was less agreement above this level. Within that range, 95% of the assays results reported in IU were within a 2.5 fold range (SD 0.4 log<sub>10</sub>), consistent with an expected variation of four times the assay variation (SD of 0.1 log<sub>10</sub>.) Cut-offs were analyzed in the light of a Robert Koch recommendation that samples with >100mIU/mL should be regarded as positive (i.e. consistent with protection.) In the study, the EQA specimen at 112 mIU/mL was reported with a range of 42-300 mIU/mL, 14% negative results and 48% equivocal results. The EQA specimen at 213 mIU/mL was reported with a range of 156-310 mIU/mL, 2% negative results and 30% equivocal results. There is a plan for future EQAs schemes for VZV IgG to include more specimens in the linear range.

**Anti-HBs**

Anti-HBs was reported in mIU/mL in 10 assays, resulting in 300-400 sets of results for the nine EQA specimens which spanned a range of ca. 10-300 mIU/mL of anti-HBs. The reference preparation was a purified human Ig and the EQA samples were obtained from vaccinees. There was 100% agreement on negativity of a negative EQA specimen (defined as <5 mIU/mL.) An upper limit of linearity of the assays was observed at 200 mIU/mL with non-commutability above this level. Agreement was investigated for EQA specimens with titers near the recommended clinical cut-off (i.e. protective level) of 10mIU/mL. For the EQA specimen at a median value of 13 mIU/mL, the results ranged from 4-35, with 21% less than 10 mIU/mL. For the EQA specimen at a median value of 18 mIU/mL, the results ranged from 8-37, with 3% less than 10 IU/mL. Within the linear range, the variability of results significantly exceeded assay variability, suggesting non-comparability of the reference preparation. For the EQA specimen at a median level of 69 mIU/mL, results were reported in a range of 32-156, with 9.5% greater than 100 mIU/mL. For the EQA specimen at a median level of 82 mIU/mL, results were reported in a range of 40-195, with 26% greater than 100 mIU/mL. Patients typically receive booster doses of HBV vaccine if the anti-HBs level is between 10-100 IU/mL. The findings of non-comparable titers of EQA specimens across the assays raised questions about the assay agreement of the reference preparation, whether vaccinee serum behaves differently than serum from naturally infected persons, whether assay differences could be due to variability in host responses to the same or different virus strains, and whether apparent non-comparability could be attributed to differences in assay designs. Variability among
assays was thought likely to reflect the complexity of the anti-HBs immune response as a mixture of antibodies that might be measured differently in different assays. It was also noted that two of the more recent anti-HBs assays gave erratic results.

**Rubella IgG**

For Rubella IgG, twelve assays reported results in IU/mL resulting in 300-400 sets of results for the EQA specimens. There was 100% agreement for negativity of a negative EQA specimen (defined as <5 IU/mL.) Agreement was explored for cut-offs in the range of 10 IU/mL, thought to correlate with immune protection. For the EQA specimen at a median level of 10 mIU/mL, results ranged from 7-230, with 40% reported at <10 mIU/mL. For the EQA specimen at a median level of 12 IU/mL, results ranged from 5-38, with 20% reported at <10 IU/mL. For the EQA specimen at a median level of 15 IU/mL, results ranged from 7-65, with 1.3% reported at <10 IU/mL. For the EQA specimen at a median level of 20 IU/mL, results ranged from 10-106, with no values reported at <10 IU/mL. Linearity was shown for assay results on EQA specimens with median values below 100 IU/mL, suggesting an upper limit for agreement of assays. Overall, across the linear range, there was lack of comparability of assay results with differences of median values on the order of ten fold, demonstrating inter-assay variability well in excess of the expected SD =0.4 log10 variation (consistent with +/- 2SD of assay variation of 0.1 log10.) As in the case of anti-HBs, the findings of non-comparable titers of EQA specimens across the assays raised questions about the comparability of the reference preparation, whether vaccinee serum behaves differently than serum from naturally infected persons, whether assay differences could be due to variability in host responses to the same or different virus strains, and whether apparent non-comparability could be attributed to differences in assay designs. However, unlike the case of HBV, there is little known antigenic variation of rubella virus as an underlying explanation for the variable findings.

Discussion of the EQA studies focused on the observation that a clinical cut-off of 10 IU/mL as determined by highly variable assays for rubella IgG is not consistent with the reality that most of the population has been vaccinated and is immune. The extreme variability that was seen in antibody detection at cut-off implies that determinations of susceptibility will be highly assay dependent, resulting in many unnecessary repeat immunizations of pregnant women. It was noted that standards in the US recommend against antenatal testing of women who have a record of rubella vaccination. In France, women are not screened only if they have a record of two vaccinations. Variability of assays to measure rubella IgG actually has increased over time, perhaps reflecting changes in product regulation. More broadly, it was remarked that similar disparate results have been observed with other serological assays and that some differences may reflect lack of harmonization of assay calibrations by the various manufacturers. In the case of HBsAg, a finding of false negative test results at the level of 0.05ng/mL is to be expected given a regulatory standard for minimum detection of 0.13ng/mL.
Appendix 3

WHO CONSULTATION ON COMMUTABILITY OF WHO BIOLOGICAL REFERENCE PREPARATIONS FOR IN VITRO DETECTION OF INFECTIOUS MARKERS

WHO headquarters, Salle C, Geneva, 18-19 April 2013

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