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# **Report**

## **WHO Consultation on the characterization of BCG vaccines**

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***WORLD HEALTH ORGANIZATION***  
**Quality Assurance and Safety of Biologicals**

## **1. Introduction**

BCG vaccine is the only vaccine currently available for immunization against tuberculosis (TB) infections and has been used since the 1920s. During this time numerous sub-strains have evolved from the original strain and have been used for vaccine production. Not surprisingly, in view of the diversity of sub-strains, manufacturing processes, immunisation schedules and levels of exposure to environmental mycobacteria and virulent *Mycobacterium tuberculosis* infection, different levels of protective efficacy of BCG vaccines in adult populations have been reported [1]. Nevertheless, as there is currently no alternative, BCG will remain in use in the foreseeable future and could continue to be used long term as a prime vaccine in a Prime-Boost immunization in conjunction with new TB vaccines. Because of this, the World Health Organisation (WHO) has recognized the need to improve both the characterization of this vaccine and the assays used for its quality control, taking into account recent advances in genetics and molecular biology.

As part of the process towards the revision of the WHO recommendations for production and control of BCG vaccines, several activities have been undertaken in 2003 and 2004, such as review of current practice in the production and control and several discussions with the experts in this area. In addition, genetic characterization of final lots and working seeds of BCG vaccines has been initiated at the National Institute for Biological Standards and Control (NIBSC), Potters Bar, UK, in September 2004, as recommended by the WHO Consultation on the characterization of BCG strains, held in London in December 2003 [2]. For the purpose of the testing, samples of working seed as well as of final production lot have been requested from the manufacturers of vaccines that have been pre-qualified for the purchase through UN system. The impact that they have on the global use of BCG vaccines has been considered important, and therefore, the

information on the genetic characteristics of these vaccines should be taken into consideration in the revision of the recommendations. At this stage, the review of the work undertaken and the advice on the additional data to be generated is essential. This should result in the appropriate preparation of the broad Consultation with BCG vaccine manufacturers, National Regulatory Authorities (NRA) and other experts in production, control and evaluation of BCG vaccines in 2005 and 2006.

An informal consultation on characterization of BCG vaccines was held on the 8 – 9 Dec 2004 at WHO Headquarters, Geneva, to review progress on the genetic characterization of BCG vaccines and to discuss the application of results to the improvement of quality control assays for BCG vaccines. The meeting was opened by Dr D. Wood, Coordinator, QSB, WHO. Dr M. Roumiantzeff was appointed Chairman, and both Drs M. Corbel and M. Ho were appointed Rapporteurs. The aims of this meeting were to:

- Discuss results of the multiplex PCR testing undertaken by NIBSC
- Review on-going research to identify any further work needed on the methodology for quality control of BCG vaccines, including molecular genetic characterisation.
- Review the current WHO requirements for production and control of BCG vaccines [3, 4] and consider changes required to reflect current state-of-the-art technology, in the light of recent developments. The Drafting Group would take the suggestions forward to produce a new draft set of recommendations which, after extensive consultation, would then be submitted to the Expert Committee on Biological Standardization (ECBS) as an update of the current document.

- Consider use of International Reference in the production and control of BCG vaccines and the need for such reference in research and development.

## 2. **Molecular characterization of BCG vaccines**

Multiplex PCR [5] has been used at NIBSC for routine identity confirmation of lyophilized BCG vaccine samples and this technique was proposed as the first step for molecular characterization of BCG vaccines. Samples of working seed strain and final production lots were received from manufacturers of vaccines that have been pre-qualified for the purchase through UN system and tested by this multiplex PCR without subculture to check for variations arising during production. Results showed that for all the BCG sub-strains tested (Russian BCG-I, Tokyo 172-1, Danish 1331, Glaxo 1077 and Connaught), there was no difference observed between the working seed lot and the corresponding final filled product. However, the routine procedure applied to lyophilized BCG samples was not sensitive enough to detect low numbers of variants in the vaccine.

Whole-genome studies of *M. tuberculosis* and BCG sub-strains have led to an improved understanding of the molecular/genomic variation of these mycobacteria [6, 7, 8]. Current BCG sub-strains are genomically different from one another. Apart from deletion of genomic regions (RDs), tandem duplications (DU1, DU2) and single nucleotide polymorphisms (SNPs) were also observed in different BCG sub-strains [9, 10]. In addition, variability in gene expression profiles and at transcriptomic level in different BCG sub-strains is also under investigation.

While it is important to study the genomic variability among different BCG sub-strains, the results obtained with the genetic methods must be related to patho-physiological findings, to determine the capacity of a given product to protect against *M. tuberculosis* challenge in animal

models. This has been seen as a first step towards better understanding of the potential influence of such differences on the protective efficacy and safety in humans. In addition, this is particularly important in the development of new live recombinant BCG vaccines.

One study in Japan using a PCR targeting the RD16 region had found two different variants/genotypes (Type I and II) in the BCG Tokyo 172-1 working seed lot. Type I had the shorter RD16 band (22 bp deletion) and Type II had an RD16 band identical with those of other BCG sub-strains. As shown by a PCR method, both variants appeared to be stable after 20 passages and were constantly present in sub-cultures. There was no significant difference between the two variants in terms of protective activities against pulmonary tuberculosis infection in the guinea pig challenge model. Real-time PCR technique may be used to determine the quantity of each variant in the BCG vaccine preparations and also in the seed lot BCG for quality control.

Historically, phenotypic variation has used to determine the maximum number of passages permissible during BCG vaccine production. The new genetic tools may be useful in the future for monitoring genetic consistency of production and to throw light on the causes, mechanism and relevance of phenotypic variations.

Currently, guinea pigs are used for routine monitoring of the presence of virulent mycobacteria in BCG vaccine. This assay is very time consuming as the animals are under observation over 6 weeks after injection of BCG vaccine. A group led by Dr Y. Lopez Vidal has developed a PCR assay to detect DNA specific to virulent mycobacteria. All BCG vaccines have deletions in the RD1 region which encode both *esat-6* and *cfp-10* genes. PCRs targeting these two genes allowed discrimination between BCG and pathogenic mycobacteria and thus detection of contamination. The sensitivity of this assay ranged from 1 genome (equivalent to 1 fg DNA) for *cfp-10* primers and 1000 genomes for *esat-6* primers when using purified DNA preparations.

Further work is required to apply this PCR assay for assuring freedom from *M. tuberculosis* contamination in BCG vaccines. This new method will not only reduce the time required for testing absence of virulent mycobacteria in BCG products, but also significantly reduce the use of animals.

### **3. Potency testing of BCG vaccines**

#### **3.1. Viable count assay**

BCG vaccine contains live bacteria, though viable count is not in itself an assay of potency, it has been used as a surrogate of BCG potency. The cultural viable count assay, often known as Colony Forming Unit (CFU) test, is problematic and can present many problems for BCG vaccine manufacturers and control laboratories. Several manufacturers presented trend analysis of results of CFU tests in routine BCG vaccine production. The CFU test is very time consuming as mycobacteria are very slow growing bacteria, thus re-testing of bulk samples before formulation is usually impossible. Together with its poor reproducibility and high variability of test results, it is the main driving force for manufacturers to look for a rapid, more reproducible alternative viable count assay. An improved ATP assay developed in Denmark using bioluminescence reaction was presented as a rapid, reproducible, high precision, low cost and less laborious viable count assay alternative. However, as with many other rapid assays, poor correlation of results from the rapid assay (measuring viability) and CFU test (measuring culturability) was observed. Further studies are in hand to see if this problem can be overcome. There is concern about the importance of good correlation between viability and culturability and thus, further improvement of this ATP assay to improve this correlation is on-going. Another viable count assay developed in France measures numbers of viable (based on the ability to

hydrolyze fluorescein-diacetate) and non-viable bacteria using flow cytometry. This method can also provide data on percentage of viable versus dead bacteria ratio in a given sample. It was agreed that this should be subjected to more general evaluation.

### **3.2. Protective potency assay**

There are various animal models (such as mouse, guinea pig) available for protective potency assay of BCG vaccines. Among these models, there are different challenge routes (respiratory, intra-tracheal, intravenous, subcutaneous, intra-peritoneal) and different challenge strains (H37Rv, Erdman, Beijing) and doses of *M. tuberculosis* in use. Different end points read out in comparison with different reference BCG strains made these assays non-comparable. There is a need for standardisation, especially for the development of new TB vaccines and/ or characterisation of products from new manufacturers.

In quality control of BCG vaccine (Tokyo 172-1) in Japan, protective potency assay of guinea pig model is used for seed lot testing. The protective efficacy of the Tokyo 172-1 seed lot was compared with Danish 1331 BCG strain and a placebo as negative control. Both BCG strains protected the infected guinea pigs in terms of significantly lower bacterial loading, far fewer histological lesions in both lung and spleen tissues, greater delayed-type hypersensitivity responses to tuberculin PPD and increased body weight gain. The guinea pig model is often used in evaluating the protective potency of new TB vaccines in Japan, Europe and USA.

### **3.3. Stability of BCG vaccine viability**

Four BCG manufacturers presented thermal and shelf-life stability data for their products. All the products appeared stable within the shelf-life and required specifications. New WHO

guideline on stability of vaccines is being developed and the issue of thermal stability tested at three different temperatures will be addressed. It was agreed that monosodium L-glutamate, which is used in the freezing medium of all these BCG products, improves the stability of this vaccine in terms of viability. In addition, evacuating the air from ampoules of lyophilized BCG vaccine using either vacuum or nitrogen can further enhance the stability of this product. However, there is some concern over the safety of the opening and administration of the vaccine in ampoules. It is generally accepted that vial filling can provide good stability for the working shelf-life of this product. Coloured brown containers should be used for filling of BCG vaccine as the product is light sensitive and viability will decrease when expose to day light [11]. Once reconstituted, BCG vaccine should be stored on ice or at 4 - 8°C and use within 4 – 6 hours.

#### **4. The requirement for an International Reference of BCG vaccine**

The current International Reference Preparation of BCG vaccine is losing its viability and the stock level is low and the preparation needs replacing. Strain-specific in-house references are often used in manufacturers' and regulatory laboratories for validation of culture medium, calibration of viable count assay, or as references for protection assay and so on. Although it is certainly a requirement for a new International Reference of BCG vaccine, the purposes of this new reference should be addressed before a new one is established. Also it could be useful to consider the need of sub-strain specific references may be required for viable count assay and for consistency purposes.

## **5. Future work proposals for molecular characterization of BCG vaccine**

- A research group led by Dr M. Behr in McGill University, Canada will perform DNA microarray for both genomic and transcriptomic comparison of the five BCG strains selected (Russian BCG-I, Tokyo 172-1, Danish 1331, Moreau RDJ and Pasteur 1173-P2). Full sequencing of selected parts of genes will be carried out if required.
- Dr G. Marchal in Institute Pasteur, France and Dr. K. Haslov in Statens Serum Institute (SSI), Denmark will continue to work on the rapid viable count assays of BCG vaccine.
- Dr Y. Lopez Vidal from University of Mexico will improve the sensitivity of the PCR (e.g. targeting *cfp-10*) for detecting virulent mycobacteria in BCG vaccine.
- Drs M. Corbel and M. Ho will lead a collaborative study on PCR for strain identification in collaboration with the working groups in McGill University, Institute Pasteur, Japan BCG Laboratory. The PCR with single set primers targeting individual deletion regions for identification will also be investigated.
- An agreement on the above proposed work would be made by all participating collaborators before the study starts in April 2005.
- WHO would coordinate and fund this work towards the revision of the recommendations for production and control of BCG vaccines.

## **6. Conclusions**

### **6.1. Agreement on the use of WHO repository of BCG sub-strains**

WHO repository of BCG sub-strains, has been established at NIBSC, in 2004, with the primary purpose of better understanding of the genetic characteristics of BCG sub-strains, currently in use. It contains samples, provided by the manufacturers of vaccines that have been

pre-qualified for the purchase through UN system. These samples should be kept for future evaluation of new tests. WHO will undertake necessary steps to make appropriate arrangement for the collection of strains/ seed lots stored at SSI, former WHO repository. A list of available material including the repository of historical samples, will be submitted and further discussed between SSI and WHO.

## **6.2. Terminology to be used**

Current proposed work is intended to improve knowledge of the molecular characterization of BCG vaccines and has been summarized in the work-plan and agreed by participating collaborators and manufacturers. For the purpose of this study and further revision of recommendations, the terminology agreed at the meeting to be used for the BCG sub-strains is Tokyo 172-1, Russian BCG-I, Danish 1331, Pasteur 1173-P2 and Moreau RDJ.

## **6.3. The issues to be addressed in the revised recommendations for production and control of BCG vaccines that may require further work**

### **6.3.1. Improvement of the identity test**

The number of sub-strains used for BCG vaccine production has effectively been reduced to Russian BCG-I, Tokyo 172-1, Danish 1331, Moreau RDJ and Pasteur 1173-P2, these accounting for >90% of production. The current identity test for BCG vaccine using acid fast staining and colony morphology lacks of specificity. It is essential to develop a robust, routine assay for manufacturers and regulatory laboratories to identify different sub-strains of BCG. This will also ensure genetic consistency in production, from master seed through working seed and to final product. Though complete genome sequencing is not necessary at this stage, other approaches

such as using DNA microarray to look for SNPs, deletions and duplications, in comparison with Pasteur 1173-P2 as the reference strain, will provide a better insight of the molecular characterisation of different BCG sub-strains. Although this technique is not intended for routine use during production, it will identify regions of interest/ importance for further study. For the purpose of the revision of WHO recommendations for production and control, the improvement of the identity test should be further considered with respect to the methodology to be used as well as to factors that may influence variability of sub-strains during the production of vaccines.

### **6.3.2. Potency testing**

A need for rapid, more reproducible alternative to viable count assay was identified. The modified ATP assay on viable counts developed in SSI is currently under evaluation, testing correlation between CFU and ATP content. New rapid assays for viable count (such as the ATP assay and fluorescein-diacetate esterase assay) after validation should be put forward for international collaborative study. Protocols will be collected and evaluated for the purpose of further testing and potential applications of these assays should be defined.

The potency of different sub-strains of BCG has been demonstrated in guinea pig protection studies. However, this is not seen as a test for routine BCG vaccine quality control. It is currently used in the development of novel TB vaccines and by at least one manufacturer, for monitoring the quality of BCG seed lots. In the context of BCG vaccines, this *in vivo* assay may be useful for assessing the potential implications of molecular genetic variations in strains/sub-strains or changes in production process.

### **6.3.3. International Reference for potency testing**

There is primary interest in establishing an International Reference for BCG vaccine as a calibrant for viable counts assay. For this purpose, it will be appropriate to collect local internal standards and to test these in parallel with relevant vaccines. At this stage, it was considered that there was no need to develop/ establish an International Reference for the protection assay for BCG testing. However, a need for establishment of an International Reference Preparation for the protection assay for the purpose of testing novel TB vaccines has been recognized. Currently, a batch of BCG vaccine is used as a comparator for consistency in protective potency assays for new TB vaccines development in Europe.

### **6.3.4. Stability testing**

BCG vaccine stability data were presented by four different manufacturers. All manufacturers found the thermal stability test for BCG vaccine to be useful and appropriate for licensing. As no failures were detected by this test, its value as a lot release test was questioned. A number of factors that influence stability profile were identified, such as vial versus ampoule filling and the inclusion of monosodium L-glutamate in the freezing medium. These should be considered in the revision of the requirements. In addition, the end of shelf-life specification needs to be further considered.

## **Annex I. List of participants**

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