World Health Organization

WHO/BS/10.2148
ENGLISH ONLY

EXPERT COMMITTEE ON BIOLOGICAL STANDARDIZATION
Geneva, 18 to 22 October 2010

International collaborative study to evaluate and establish the 1st WHO Reference Reagents for BCG vaccines of Russian BCG-I sub-strain

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Introduction

The WHO/BS/09.2114 report 'International collaborative study to evaluate and establish the 1st WHO Reference Reagents for BCG vaccines of three different sub-strains' was submitted to the WHO ECBS for considerations in 2009. Two of the BCG sub-strain preparations (Danish 1331 and Tokyo 172-1) were adopted as the WHO Reference Reagents in the ECBS meeting held in Oct 2009. However, due to the absence of moisture content data for the Russian BCG-I sub-strain, the Committee agreed to establish the Russian BCG-I sub-strain on condition that 'the moisture content for the Russia BCG-I reference material be provided and reviewed and found acceptable by the Committee'. Thus this additional report has been prepared to summarise data from the collaborative study presented in the WHO/BS/09.2114 report, to present the additional moisture content and real-time stability data (also in comparison with the other two established WHO Reference Reagents for BCG vaccines) of the Russian BCG-I sub-strain preparation.

Five thousand ampoules of lyophilised BCG vaccine of Russian BCG-I sub-strain were generously donated by BB-NCIPD Limited (an established BCG vaccine manufacturer which is pre-qualified by WHO) to WHO. Samples of this BCG sub-strain were included in the collaborative study for the evaluation of candidates for WHO Reference Reagents for BCG vaccines of different sub-strains. The collaborative study involving eleven participating laboratories in nine countries was performed in 2008/9 and included two viability assays (cultural viable count and ATP assays) to evaluate the content of these preparations and the multiplex PCR as identity test. The summary results of BCG Russian BCG-I sub-strain preparation are extracted from the WHO/BS/09.2114 document and presented in this report as reference.

As the manufacturer did not perform any assays for the moisture content of this preparation, no data for moisture content were submitted. The moisture content of the BCG Russian preparation was determined at NIBSC in November 2009 using the coulometric Karl Fischer titration method.

BCG vaccine is a live bacterial preparation and its stability is monitored by cultural viable count assay in terms of CFU/ ampoule. It was agreed in the ECBS meeting in October 2009 that routine accelerated degradation study for this type of reference material will not be suitable. Thus real-time stability study using cultural viable count assay with material stored at -20°C will be carried out at NIBSC for the next 10 years with one test per year to ensure the viability of the preparations maintained within the acceptable range (as estimated from this collaborative study). Any significant reduction of the cultural viable count results (as viability) from these Reference Reagents should be reported in the Instruction for Use to notify the end users. Additional data of real-time stability for BCG Russian BCG-I sub-strain are presented in this report. The real-time stability data for the other two established WHO Reference Reagents for BCG vaccines (Danish 1331 and Tokyo 172-1) are also included in this report for comparison.

The aim of this additional report is to provide additional data requested by WHO ECBS and to propose the establishment of the Russian BCG-I sub-strain preparation as the 1st WHO Reference Reagent for BCG vaccine of Russian BCG-I sub-strain.

Results

Moisture content of BCG Russian BCG-I sub-strain

Ten ampoules of BCG vaccine (Russian BCG-I sub-strain) reference material (NIBSC code 07/274) were subjected to moisture content determination by coulometric Karl Fischer titration method at NIBSC in November 2009. Due to the low dry mass (assumed 3.5 mg), and

difficulties with the non-NIBSC ampoule format, measurements were made on both single ampoules and on three ampoules assayed simultaneously. Both methods yielded a moisture content of 5 - 6% and the mean of the 4 determinations was 5.4% w/w.

Table 1. Results of moisture content estimation of BCG Russian BCG-I sub-strain preparation using Karl Fischer titration method

Estimation	Number of ampoules tested	Residual moisture % w/w
1	1	5.67
2	1	4.73
3	3	5.24
4	3	5.76
	Mean moisture content	5.37% SD = 0.49% CV = 9.2%

Key: w/w = weight per weight; SD = standard deviation; CV = Coefficient of variation

The high level of moisture compared to typical WHO International Standards may be due to a number of factors. The coulometric Karl Fischer method usually gives higher than expected moisture content when the dry weight is below 10 mg/ ampoule. However, results were very similar whether ampoules were assayed singly or after pooling three ampoules at a time. The product was a powder rather than a well formed freeze dried cake and so might have more associated moisture. The majority of the material was sodium glutamate – this may be crystalline and so have tightly bound water of hydration with it. The moisture content of this product does seem to be genuinely higher than for other lyophilised WHO standards and it is noted that it is possible to lose viability of live bacteria due to over-drying.

Summary of CFU and ATP content of BCG Russian BCG-I sub-strain

Data extracted from the collaborative study presented in the WHO/BS/09.2114 report. Results from the two other WHO Reference Reagents for BCG vaccines are also included as for comparison.

Table 2. Summary of results from cultural viable count assays calculated using the method described in WHO/TB/Technical Guide/77.9. The mean content of each BCG Reference Reagent candidate is presented as CFU x 10^6 / ampoule.

	Danish sub-strain			**Russian sub-strain**			Tokyo sub-strain		
Laboratory	Mean	SD	CV	Mean	SD	CV	Mean	SD	CV
1	3.70	0.45	12.2%	3.07	0.29	9.4%	24.08	2.45	10.2%
2a	7.89	1.14	14.4%	3.54	0.17	4.8%	40.07	3.08	7.7%
2b	8.96	0.51	5.7%	2.67	0.29	10.9%	55.95	1.68	3.0%
3	8.70	2.05	23.6%	2.61	0.21	8.0%	60.07	1.13	1.9%
5	4.77	0.73	15.3%	1.44	0.25	17.4%	39.84	3.16	7.9%
6	12.68	2.46	19.4%	4.01	0.60	15.0%	65.33	10.86	16.6%
7	3.02	0.88	29.1%	1.34	0.37	27.6%	16.89	3.49	20.7%
9	8.30	2.10	25.3%	3.76	0.92	24.5%	55.82	9.78	17.5%
10	7.31	0.71	9.7%	4.84	2.18	45.0%	61.43	6.27	10.2%
11	7.59	2.02	26.6%	6.63	1.13	17.0%	74.22	8.05	10.8%
Mean	**7.29**			**3.39**			**49.37**		
SD of mean	0.90 (12.3%)			0.50 (14.7%)			5.86 (11.9%)		
Pooled between-ampoule SD	1.48 (20.4%)			0.95 (28.1%)			6.59 (13.1%)		
Combined uncertainty	**1.74 (23.8%)**			**1.08 (31.8%)**			**8.74 (17.7%)**		
Expanded uncertainty (95% confidence)	**3.37 – 11.22**			**0.95 – 5.83**			**29.60 – 69.14**		

Key: SD= Standard deviation; CV= Coefficient of variation

Table 3. Summary results of modified ATP assay. The mean ATP content is presented as ng/ ampoule.

	Danish sub-strain (tested neat)			**Russian sub-strain** (tested neat)			Tokyo sub-strain (tested diluted 1:5)		
Lab	Mean	SD	CV	Mean	SD	CV	Mean	SD	CV
1	34.33	6.50	18.9%	4.11	0.88	21.4%	124.25	6.95	5.6%
3	99.32	23.82	24.0%	14.38	8.29	57.7%	322.16	27.19	8.4%
5	69.09	7.23	10.5%	10.46	3.82	36.5%	271.19	18.49	6.8%
6	48.84	4.76	9.8%	4.86	0.64	13.2%	173.52	14.23	8.2%
8	75.16	53.54	71.2%	15.38	9.06	58.9%	502.34	266.63	53.1%
9	61.58	14.21	23.1%	8.74	2.36	27.1%	279.99	42.39	15.1%
10	41.77	10.61	25.4%	6.46	2.26	35.0%	174.81	73.54	42.1%
11	37.48	12.90	34.4%	3.62	1.05	29.0%	177.28	20.47	11.5%
Mean	**58.45**			**8.50**			**253.19**		
SD of mean	7.87 (13.5%)			1.62 (19.0%)			42.84 (16.9%)		
Mean*	**56.06**			**7.52**			**217.60**		
SD* of mean	8.65 (15.4%)			1.48 (19.7%)			27.52 (12.6%)		
Pooled between-ampoule SD*	12.57 (22.4%)			3.49 (46.4%)			35.62 (16.4%)		
Combined uncertainty*	**15.26 (27.2%)**			**3.79 (50.4%)**			**45.01 (20.7%)**		
Expanded uncertainty* (95% confidence)	**18.71 – 93.40**			**0 – 16.79**			**107.47 – 327.74**		

Key: SD= Standard deviation; CV= Coefficient of variation; *=results from Laboratory 8 excluded in the calculation of mean, SD and uncertainty.

Real-time stability data of BCG Russian BCG-I sub-strain

A real-time stability programme to monitor the cultural viable counts of the BCG RR candidates in storage at -20°C in a yearly basis (one cultural viable count assay per year) has been established. The assays will be performed at NIBSC only. Three assays have been performed in 2008/ 9 (at 4 - 5 months intervals) to establish a base line and this will be used to assess the viability in terms of CFU of each preparation over the next 10 years. Figure 1 showed the CFU content of BCG Russian BCG-I preparation from 2008 to 2010. This result is also compared with the CFU results of the other two established WHO Reference Reagents for BCG vaccines (Danish 1331 and Tokyo 172-1 sub-strains). The upper and lower limits of each sub-strain preparation are estimated from the expanded uncertainty (95% confidence) of the collaborative study on cultural viable count (Table 2). The results showed that the cultural viable counts of all three preparations were well within the acceptable range specific for each product.

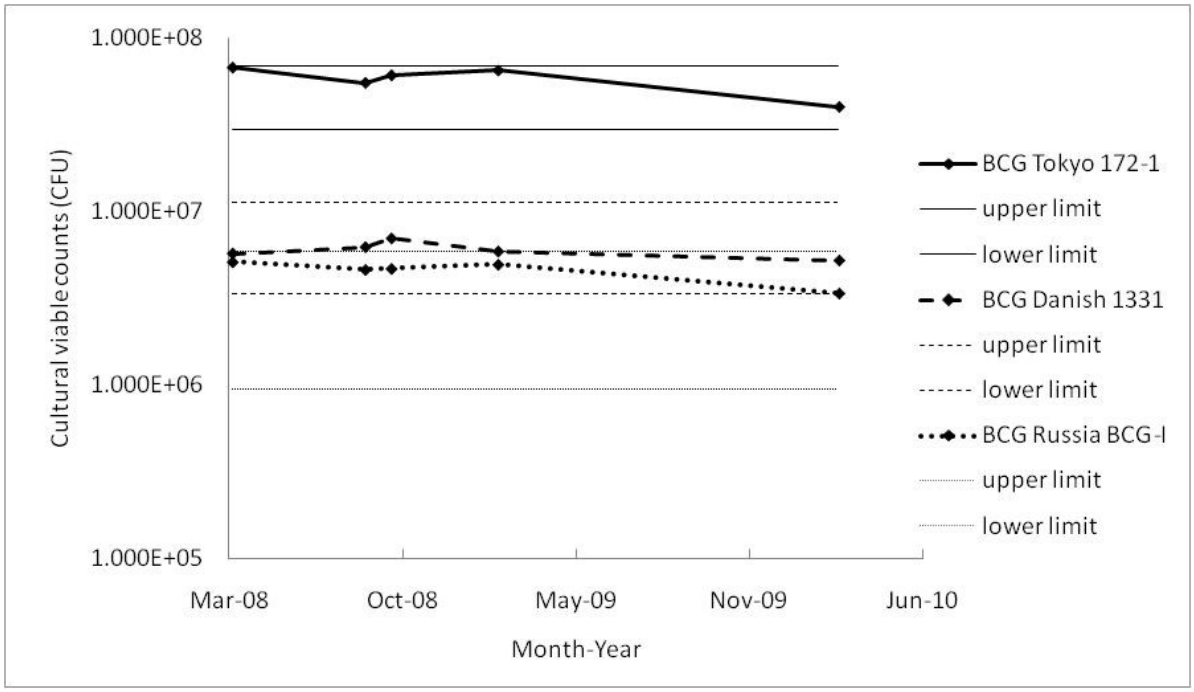

Figure 1. Real-time stability data on cultural viable count assay of WHO Reference Reagents for BCG vaccines of different sub-strains. Assays were performed at NIBSC only. Upper and lower limits of each sub-strain are estimated from the expanded uncertainty (95% confidence) from the collaborative study (Table 2).

Discussion

Two sets of new data are presented in this report to support the proposal to establish the BCG Russian BCG-I sub-strain preparation as a WHO Reference Reagent. The moisture content of this preparation was estimated as 5.4%. It is relatively higher than the other two WHO Reference Reagents for BCG vaccines (3.1% for Danish 1331; 2.9% for Tokyo 172-1). However, there is no specification for the residue moisture content of lyophilised BCG products in WHO Requirements for dried BCG vaccine nor the European Pharmacopoeia monograph on BCG vaccine, freeze-dried. In addition, there is no final product specification on moisture content for this BCG Russian BCG-I sub-strain preparation, and the manufacturer has not tested this. Thus there is no information that the 5.4% moisture content of this preparation is high for this product and thus it is speculative whether it will have any negative impacts on the long-term stability in terms of viability on this preparation.

Despite of the recent development of various alternative viability assays, the cultural viable count assay remains as a 'gold standard' to determine the viability of lyophilised BCG vaccine as cultural particles or CFU. The results from cultural assay can be variable due to various factors including clumping in the suspension, change of operators, any subtle changes in culture medium and reagents from the suppliers. Thus the stability of a given BCG preparation is considered unchanged if the CFU counts are within an acceptable range (with upper and lower limits) specific for the preparation. The real-time stability study is to monitor the CFU counts of the WHO Reference Reagents for BCG vaccines to ensure the viability of these preparations remains within the acceptable range. From 2008 to 2010, the CFU counts of BCG Russian BCG-I preparation were well within its upper and lower limits. Thus its viability is as stable as the other two established WHO Reference Reagents for BCG vaccines (Danish 1331 and Tokyo 172-1) when stored at -20°C.

Proposal

This proposal is for the establishment of the batch of ampoules coded 07/274 as the First WHO Reference Reagent for BCG vaccine of Russian BCG-I sub-strain. Based on the results from the cultural viable count and ATP assays, the content estimated in the collaborative study performed in 2008/9 for BCG Russian BCG-I (NIBSC code: 07/274) is 3.39 million CFU or 7.52 ng ATP per ampoule. Due to the relatively large variations in the estimation of the content of this preparation, the preparation is not recommended to be used as a calibration standard. The unitage values should be used as a guide for the end users who may need to estimate the content of the preparation in their own assays. Any significant reduction of CFU below the lower limits detected in the future will be reported in the Instruction for Use which is issued with all reference material distributed by NIBSC to the end users. The intended uses of these proposed RRs for BCG vaccine are as comparators for viability assays (such as cultural viable count and modified ATP assays), residual virulence/ local reactogenicity assays and protection assay in animal model; and as Reference BCG sub-strains for identity assay using molecular biology techniques.

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