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Model Systems

Comparison of pellicle and shake flask-grown BCG strains by quality control assays and protection studies

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ABSTRACT

A global BCG vaccine shortage began in 2013 which impacted availability for infant vaccinations, as well as preclinical studies and clinical trials of new TB vaccines. Stakeholders met in 2015 at McGill University in Montreal to discuss the shortage and potential mitigation strategies. Manufacturing BCG through a more tractable liquid fermentation process instead of the traditional pellicle growth method was considered a potentially viable strategy. This pilot program compared pellicle-grown and shake flask-grown BCG strains (as a first step towards modeling fermenter-produced BCG vaccine) in selected quality control assays, as well as mouse and guinea pig protection studies. Conventional pellicle-grown, lyophilized BCG WHO Reference Reagents (Danish, Moreau, Russian, Tokyo strains) were obtained from the National Institute for Biological Standards and Control (NIBSC), UK. Strains were grown in shake flasks and glycerol stocks prepared. Shake flask-grown BCG culture preparations generally met the requirements of quality control testing at NIBSC. In mouse and guinea pig protection studies there were no significant differences in lung colony forming units (CFUs) between shake flask-grown and pellicle-grown preparations, with the exception of BCG Russian, where the shake flask-grown preparation protected better in mice ($P = 0.0042$), but the pellicle-grown preparation protected better in guinea pigs ($P = 0.0015$). Producing BCG vaccines by a more tractable liquid growth process could be a viable solution to the global BCG shortage.

1. Introduction

BCG, the world's most widely used vaccine [1], prevents extrapulmonary childhood tuberculosis (TB), and routine immunization in high endemic countries is recommended by the World Health Organization (WHO) [2]. Unfortunately, a global BCG vaccine shortage began in 2013, with the United Nations Children's Fund (UNICEF), the main supplier of BCG vaccine to TB-endemic countries, reporting shortages of approximately 8 million doses. By 2015, the shortfalls reached 16.5 million doses [3], which was estimated to translate to 7433 excess TB deaths in the pediatric population (less than 15 years of age) [4]. While increased country requirements and buffer stock replenishments were partially to blame, the shortages were largely caused by production issues, when two of UNICEF's four suppliers experienced technical manufacturing difficulties [3]. These shortages impacted the availability of BCG vaccine for infant vaccination and obliged some countries to seek alternative manufacturers for the administration of BCG vaccine that was not licensed in the home country [5].

Both preclinical studies and clinical trials of new TB vaccines were also affected. Licensed BCG vaccine was appropriately prioritized for infant vaccination. This resulted in disruptions to preclinical research and clinical trials until alternative sources of BCG vaccine could be accessed for *in vitro* experiments, animal studies, and as controls in clinical trials. One phase IV trial comparing the efficacy of two BCG sub-strains was even forced to switch strains mid-trial due to the shortage [6]. As the need for BCG vaccine had no end in sight, the BCG vaccine shortage was expected to continue unless appropriate interventions were made [3]. Manufacturers, academics, and product developers met at McGill University in Montreal in October 2015 to explore strategies to alleviate the shortage. One such strategy included

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manufacturing BCG using a process with higher yield, less complexity, and more reproducibility than the existing licensed vaccine production methodology.

All currently licensed BCG vaccines are produced using a pellicle growth method [7]. Utilizing this method, BCG vaccine strains are cultured in stationary flasks, in which a pellicle, or floating biofilm, of BCG is formed. The pellicle is then collected, milled, filled and lyophilized. However, this method is cumbersome and difficult to standardize [8,9], leading to issues with GMP compliance. These compliance issues have contributed to manufacturers' inability to meet demand. An alternative approach is to grow BCG by fermentation prior to lyophilization. Fermentation has the potential to overcome issues seen with the traditional pellicle growth method, however such a substantial change in the manufacturing process, from a regulatory perspective, would lead to the BCG being considered a new product, with the potential requirements for extensive clinical studies since the possibility exists that fermenter-grown BCG may have quality attributes different from those that are pellicle grown, potentially impacting the safety, immunogenicity, or effectiveness of the vaccine.

The studies presented here compare sub-strains of pellicle-grown, lyophilized BCG to shake flask-grown, glycerol stocks of the same sub-strain. In this pilot study we consider shake flask-growth a reasonable substitute for the fermentation process as both methods use agitation, a similar growth medium containing detergent, and the use of air exchange (as the BCG fermentation process is not anaerobic). The comparison studies presented include those outlined by the BCG vaccine monograph of the European Pharmacopeia [10], including viable bacterial count, identity by PCR, excessive dermal reactivity, absence of virulent mycobacteria, and delayed hypersensitivity, as well as protection studies in the mouse and guinea pig models of TB to evaluate relevant biological activities. These comparisons were performed separately using the four well-characterized WHO Reference Reagent of BCG vaccine strains distributed by NIBSC. Results indicate that products from both manufacturing methods provide similar outcomes in terms of safety, quality and in animal protection models.

2. Materials and methods

2.1. Animals and ethical considerations

All animal procedures were approved by the respective local ethics committees at PHE Porton and NIBSC and were in accordance with UK Home Office (Scientific Procedures) Act 1986. Animals were monitored by trained animal technicians at least once a day, and this frequency could increase if any adverse reactions were observed. All efforts were made to avoid bias e.g. using unique animal identification codes for random allocation of animals to groups and performing analyses in a blinded manner. Group size for the guinea pig protection studies was determined by statistical power calculations (Minitab version 16) with the aim to reliably detect (with 95% confidence) a difference between the median colony forming units (CFU) per ml of 1.0 $\log_{10}$.

2.2. BCG strains

The pellicle-grown, lyophilized WHO reference reagents of four BCG sub-strains were obtained from NIBSC (Potters Bar, Hertfordshire, UK; WHO International Laboratory for Biological Standards, UK Official Medicines Control Laboratory). These include BCG Danish (NIBSC code: 07/270), BCG Moreau (NIBSC code: 10/272), BCG Russian (NIBSC code: 07/274), and BCG Tokyo (NIBSC code: 07/272). Aeras prepared the shake flask-grown BCG test preparations. The four lyophilized BCG reference reagents were reconstituted and cultured at 37 °C in 7H9 (BD Middlebrook) growth medium containing 0.05% tyloxapol (Sigma) and free of animal products. Cultures were expanded from 5 to 10 mL in 30 mL PETG square bottles (Nalgene). Once cultures were turbid, they were expanded to 20 mL in 1 L roller bottles (Corning) and grown at 37 °C on roller rack until an OD_{600} of approximately 2 was reached. Cultures were then expanded to their final volume in 500 mL baffled-bottom Erlenmeyer flasks (VWR) and grown at a shaking speed of approximately 125 rpm. Cultures were concentrated two-fold in a glycerol freezing buffer when an OD_{600} of approximately 4 was reached. Concentrated cultures were then vialed in cryovials (Corning), and stocks were stored at −80 °C.

2.3. Culture viable count assay

The viable counts of each of the four, shake flask-grown BCG test preparations were determined by a culture plating method. Dubos agar supplemented with oleic acid and horse blood was prepared in-house at NIBSC and used as the solid culture medium in this test. The shake flask-grown BCG test preparation glycerol stocks were thawed at room temperature and serially diluted using a sterile sample diluent (0.5% v/v tyloxapol in deionized water), and plated in triplicate onto the agar plates. The plates were incubated at 37 °C for 3 weeks, and colonies were counted for determination of CFUs per mL. A lyophilized BCG reference reagent with known CFUs per ampoule was used as a relevant control for assay performance.

2.4. Identity test using multiplex PCR (mPCR)

A multiplex PCR assay was used to confirm the identity of the four, shake flask-grown BCG test preparations. Genomic DNA was extracted and purified from each BCG test preparation and reference reagent using the GenElute Bacterial Genomic DNA Kit (Sigma, UK). The purified genomic DNA was used in PCR reactions with a set of 13 primers designed to amplify specific regions within the BCG genome that would identify specific BCG sub-strains [11]. Details of experimental conditions were described previously [12]. In brief, the mPCR was performed on extracted DNA samples with the 13-primer set using a thermal profile of 1 cycle at 94 °C (10 min), 30 cycles at 94 °C (1 min), 55 °C (1 min) and 72 °C (2 min), and 1 cycle at 72 °C (10 min). The mPCR products were then analyzed by horizontal electrophoresis on 3% (w/v) agarose gels containing SafeView DNA stain (Applied Biological Materials Inc) in TAE buffer according to standard methodologies, and resulting PCR product profiles were compared to the corresponding reference reagent.

2.5. Excessive dermal reactivity in Guinea pigs

For each of the four BCG sub-strains, a group of six female Dunkin Hartley guinea pigs (250–400 g) was used (24 animals total). Each guinea pig was injected intradermally with 0.1 mL of 3×10^6 CFU/mL (BCG single human dose), 3×10^5 CFU/mL, and 3×10^4 CFU/mL of the shake flask-grown BCG test preparation, as well as identical doses of the corresponding reference reagent. A randomization plan was used to determine the injection sites for the doses of test preparation and reference reagent (front, middle, or hind on either left or right flank of each guinea pig). Lesions formed at the site of injection were observed for 4 weeks and their body weights were measured at regular intervals (weekly). In each animal, the papule sizes induced by the test sample were compared with those induced by the reference reagent.

2.6. Absence of virulent mycobacteria in Guinea pigs

For each of the four, shake flask-grown BCG test preparations, a group of six female Dunkin Hartley guinea pigs (weight range 250–400 g) was used (24 animals total). Each guinea pig was injected intramuscularly with 0.1 mL of 1.5×10^8 CFU/mL, (equivalent to 50x the single human dose of BCG). Animals were weighed at regular intervals (weekly) and observed daily for 42 days for any TB symptoms. At the end of this period, the animals were euthanized and examined by necropsy for signs of infection with *Mycobacterium tuberculosis* (Mtb).

2.7. Delayed hypersensitivity in Guinea pigs

For each of the four BCG sub-strains, a group of 6 female Dunkin Hartley guinea pigs (weight range 250–400 g) was used for both the shake flask-grown test preparations and the reference reagents (48 animals total). Each guinea pig was injected intradermally with a single human dose of BCG (0.1 mL of 3×10^6 CFU/mL). Animals were weighed at regular intervals (weekly). After 28 days post immunization, animals were injected intradermally with PPD (WHO International Standard of Tuberculin PPD diluted in sterile saline to 10 IU/100 μL). The induration formed at the site of injection was observed and recorded at both 24 and 48 h. The induration observed from the test preparations was compared with that observed from the reference reagents of the corresponding BCG sub-strain. The Student's *t*-Test was used for statistical analysis ($P < 0.01$).

2.8. Protection in the murine aerosol challenge model

For each of the eight BCG groups (four shake flask-grown BCG test preparations and four corresponding pellicle-grown BCG reference reagents) and two control groups (commercial SSI BCG Danish 1331 control and saline), 8 Balb/c mice were used (80 animals total, plus five untreated mice to determine challenge uptake). All BCG groups were vaccinated with 3×10^5 CFU/mouse, administered in 2×25 μL injections by bilateral intradermal injections at week 0. Four weeks post vaccination, mice were aerosol challenged with low dose Mtb H37Rv using a Nebulizer System (Walkers, UK) linked to a Middlebrook airborne infection device (Glas-col, Terre Haute, USA). Mtb H37Rv stock was diluted to approximately 5.0×10^6 CFU/mL for the aerosol generation to achieve an estimated challenge dose of approximately 100 CFU/lung. Four weeks post challenge, animals were necropsied and lungs and spleen were harvested for CFU determination. Lung lobes and spleen from each animal were removed and homogenized, and samples were serially diluted and plated in duplicate onto OADC-supplemented 7H11 agar. The plates were incubated at 37 °C. Bacterial colonies were enumerated after 2–3 weeks. The Mann-Whitney test was used for statistical analysis ($P < 0.01$).

2.9. Protection in the Guinea pig aerosol challenge model

Individual guinea pigs in the protection study were randomly assigned to vaccine groups and identified using subcutaneously implanted microchips (PLEXX BV, The Netherlands). For each of the eight BCG groups (four shake flask-grown BCG test preparations and four corresponding pellicle-grown BCG reference reagents) and two control groups (commercial SSI BCG Danish 1331 control and saline), 10 female Dunkin Hartley guinea pigs (300–400 g), free from pathogen-specific infection, were used (100 animals total). All BCG groups were subcutaneously vaccinated with BCG (0.25 mL of 5×10^4 CFU/guinea pig) at week 0, and challenged with approximately 20–50 CFU of Mtb H37Rv (NCTC 7416) via the aerosol route at 10 weeks post vaccination. Animals were challenged using a Henderson apparatus in conjunction with an AeroMP control unit, as previously described [13–15]. Four weeks post challenge, animals were necropsied and lungs and spleen were harvested for CFU determination (as described for the murine challenge model) and histopathology scoring. Tissue representative of each lung, sampled consistently between animals, was processed routinely (by formaldehyde fixation) and embedded in paraffin wax. Sections (thickness, approximately 5 μm) were stained with hematoxylin and eosin. The nature and severity of the lesions were assessed by a blinded investigator, using a subjective scoring system. Each lung lobe was assigned a score as previously described [16]. Scores from each lobe were combined. A mean score from lung lobes was calculated for each group. Group mean histopathology scores were compared between groups and with bacterial loads. The Mann-Whitney test was used for statistical analysis ($P < 0.05$).

Table 1
Culture viable count determinations for shake flask-grown BCG test preparations.

BCG Sub-strain	Culture Viable Count Determination[a] (x 10^8 CFU/mL)
Danish 1331	1.88 ± 0.19
Moreau-RJ	6.57 ± 0.91
Russian BCG-I	5.85 ± 0.95
Tokyo 172	10.7 ± 0.91

[a] The culture viable count determination is the average of six estimates obtained in two separate assays performed at NIBSC, and was used when calculating doses for the other assays included in this study.

2.10. Statistical analyses

Statistical tests were performed using Minitab version 16 (power and sample size calculations) or Graph Pad Prism version 7 (analyses of differences between BCG vaccines).

3. Results

Each of the selected quality control assays (Supplementary Table 1) was performed on the four, shake flask-grown BCG test preparations and their pellicle-grown reference reagent counterparts. The CFU titers obtained from the culture viable count assay (Table 1) were used for dosing calculations for the additional assays performed.

3.1. Identity test using multiplex PCR (mPCR)

The mPCR products from all shake flask-grown BCG test preparations and pellicle-grown BCG reference reagents were detected at the expected sizes (Supplementary Table 2 and Fig. 1) except for the shake flask-grown BCG Tokyo test preparation. BCG Tokyo is known to contain two variants, 172-I (Type I) and 172-II (Type II), where the two types differ in Region of Difference 16 [17,18]. The mPCR assay distinguishes between the two variants by producing PCR products of 379 bp and 401 bp for Type I and Type II, respectively. While the pellicle-grown reference reagent had a PCR profile that was largely Type I, the corresponding shake flask-grown BCG test preparation was predominantly Type II (Fig. 1a and b).

3.2. Excessive dermal reactivity in Guinea pigs

This safety test was used to determine any excessive dermal reactivity of the shake flask-grown BCG test preparations. Shake flask-grown preparations complied with the test if papule sizes were not markedly different from the corresponding pellicle-grown reference reagents. All animals were well and healthy throughout the experiment. They all had a gradual body weight increase over the 4 weeks' period. A gradual reduction of the average papule size was observed in both the shake flask-grown BCG test preparations and the pellicle-grown BCG reference reagents when they were serially diluted tenfold. Shake flask-grown Danish and Tokyo BCG preparations appeared to have slightly less dermal reactivity than their corresponding reference reagents, while shake flask-grown Moreau and Russian BCG preparations appeared to have slightly higher dermal reactivity than their corresponding reference reagents. Any observable differences were within the acceptable range of papule sizes (maximum at 5.0 mm) as detected using the BCG reference reagents in this experiment (Fig. 2). All of the shake flask-grown BCG test preparations complied with the excessive dermal reactivity test.

3.3. Absence of virulent mycobacteria in Guinea pigs

This safety test was used to ensure the shake flask-grown BCG test preparations did not contain any residual virulent mycobacteria. Shake

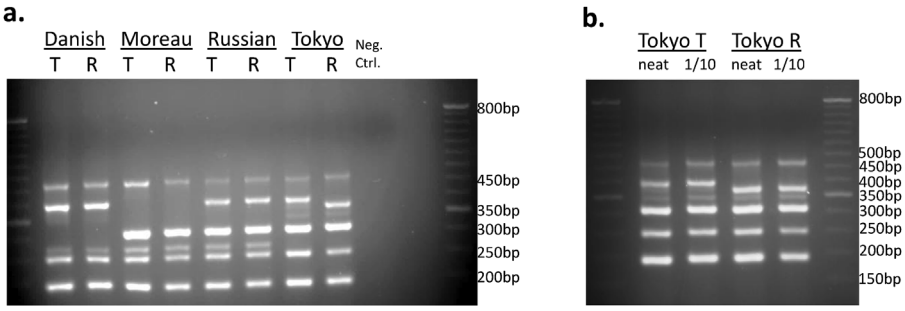

Fig. 1. Multiplex PCR (mPCR) fingerprints of BCG test and reference preparations. BCG sub-strain-specific targets were chosen for amplification to compare profiles of the shake flask-grown test preparations (T) with the pellicle-grown reference reagents (R). The resulting PCR products were analyzed by electrophoresis with a 3% agarose gel containing SafeView DNA stain (a). Size differences were found in the BCG Tokyo mPCR assay, where the shake flask-grown test preparation yielded a 401 bp PCR product for RD16 and the pellicle-grown reference reagent yielded a 379 bp PCR product, indicating that the strains were two different types of BCG Tokyo, 172-2 and 172-1, respectively (b). Molecular size markers are shown on the left and right lanes in each gel.

flask-grown preparations complied with the test if none of the animals showed signs of TB symptoms, if the animal gained weight and autopsy did not reveal any sign of TB infection, and if not more than one animal per group died during the observation period. All animals were well and healthy without any abnormal behavior throughout the experiment. They all had a steady increase in body weight over the 6 weeks' period. Post-mortem examination of all guinea pigs at Day 42 showed no abnormalities in the vital organs and the animals showed no signs of TB-like lesions in the lungs, spleen, kidney or liver (data not shown).

3.4. Delayed hypersensitivity in Guinea pigs

This potency test was used to determine the biological activity of the shake flask-grown BCG test preparations. Shake flask-grown preparations complied with the test if the size of the skin reaction following injection of PPD was not significantly different from the comparison reference reagent of the same sub-strain. All animals were well and healthy throughout the experiment. They all had a gradual body weight increased over the 4 weeks' period. Sizes of induration observed in all animals ranged from 10 to 20 mm at 24 h (Day 29), or from 8 to 18 mm at 48 h (Day 30) post PPD injection (Fig. 3). The average sizes of induration decreased slightly at Day 30 in all groups as expected. Only shake flask-grown BCG Danish was statistically significantly more potent in the delayed hypersensitivity test compared with the pellicle-grown BCG Danish reference reagent ($P = 0.001$). All other shake flask-grown BCG test preparations complied with the delayed hypersensitivity test.

3.5. Protection in murine aerosol challenge model

This assay was performed to compare the level of protection induced by shake flask-grown BCG test preparations with the pellicle-grown BCG reference reagent counterparts in a mouse aerosol challenge model. Following aerosol challenge with Mtb H37Rv, the uptake challenge dose was estimated at an average of 32.4 CFU/lung. At 4 weeks post challenge the level of infection observed in the lungs of saline control mice was approximately 1.16×10^6 CFU/lung. This confirmed that the infection was successfully established. Mice vaccinated with a single dose of the BCG SSI reference control had approximately a 1 log reduction in CFUs in their lungs compared to the saline control group, confirming previous experience of the behavior of the assay.

In the lung, the shake flask-grown BCG test preparations provided similar protection to that offered by the pellicle-grown reference reagents (Fig. 4a). The exception was shake flask-grown BCG Russian, which had statistically significantly lower lung CFU when compared with the pellicle-grown BCG Russian reference reagent ($P = 0.0042$). No statistically significant differences in spleen CFU were seen between test preparations and reference reagents (Fig. 4b).

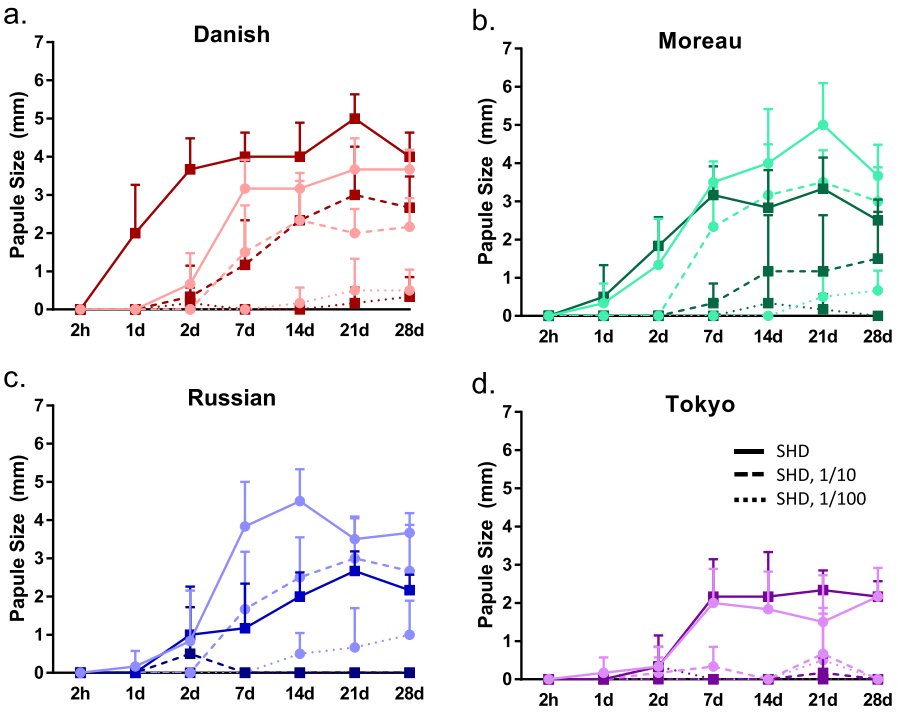

Fig. 2. Excessive dermal reactivity in guinea pigs. For each of the four BCG sub-strains (a. Danish, b. Moreau, c. Russian, d. Tokyo), a group of six female Dunkin Hartley guinea pigs were used. Each guinea pig was injected with shake flask-grown test preparation (●) or pellicle-grown reference reagent (■). Each animal received intradermal injections with a single human dose (SHD) of BCG (0.1 mL of 3×10^6 CFU/mL), as well as 1/10 and 1/100 dilutions of the SHD. Lesions formed at the sites of injection were observed over a 28-day period. In each animal, the papule sizes resulting from the shake flask-grown test preparation were compared with those resulting from the pellicle-grown reference reagent.

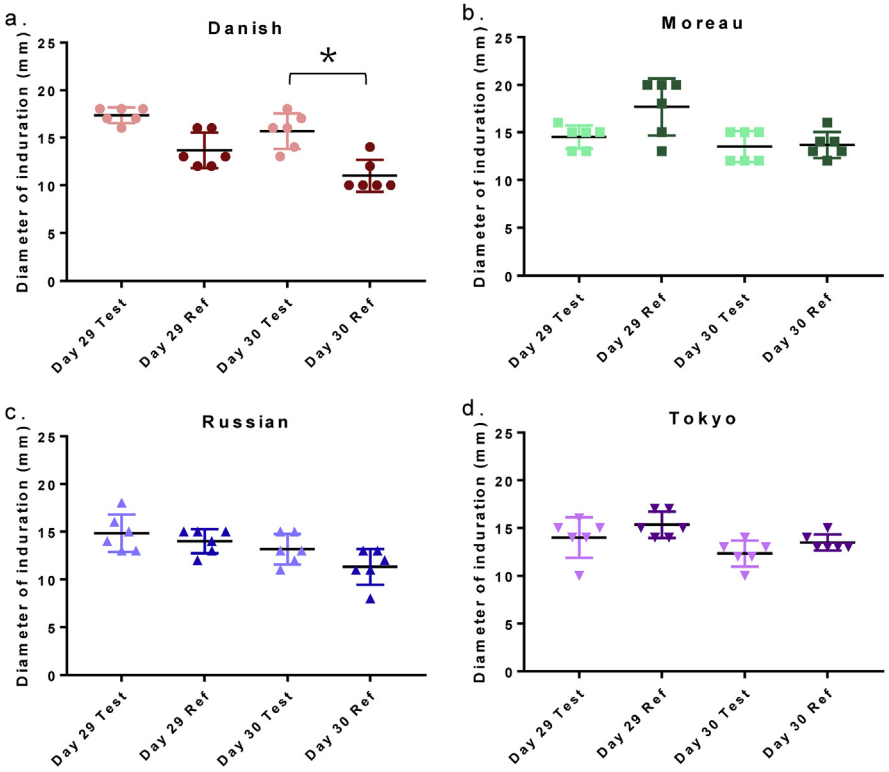

Fig. 3. Delayed hypersensitivity in guinea pigs. For each of the four BCG sub-strains (a. Danish, b. Moreau, c. Russian, d. Tokyo), 6 female Dunkin Hartley guinea pigs were used for either the shake flask-grown test preparation or the reference reagent. Each guinea pig was injected intradermally with a single human dose of BCG (0.1 mL of 3×10^6 CFU/mL), and after 28 days injected intradermally with Tuberculin PPD. The diameter of induration at injection site of PPD was measured and recorded at Day 29 (24 h post PPD injection) and Day 30 (48 h post PPD injection). Individual data points with the mean are shown. $^*P < 0.01$ by the Student's *t*-Test (two-sample equal variance with two-tailed distribution; $P = 0.001$).

3.6. Protection in Guinea pig aerosol challenge model

For the guinea pig protection studies, all BCG strains tested (both shake flask-grown test preparations and pellicle-grown reference reagents) gave comparable protection in both tissues except for BCG Russian, where the pellicle-grown reference reagent was statistically significantly better in the lung than the shake flask-grown BCG Russian test preparation ($P = 0.0015$) (Fig. 5a), and BCG Danish, where the shake flask-grown test preparation offered statistically significantly better protection in the spleen than the pellicle-grown BCG Danish reference reagent ($P = 0.0261$) (Fig. 5b).

All vaccine groups had a significantly lower group mean lung histopathology score, compared with the unvaccinated control group (group mean histopathology score of 18.4), and the severity of microscopic lesions was similar between the BCG vaccinated groups (group mean histopathology scores range between 3.45 and 7.7, and there was little necrosis). In the spleen, no lesions were observed in groups that had been given shake flask-grown BCG Danish or BCG Russian. The remaining BCG vaccinated groups had a lower group mean pathology score than the unvaccinated control group and the number of lesions in the spleen were similar between the BCG vaccinated groups (group mean histopathology scores ranged between 0 and 0.6). Data not shown.

4. Discussion

The results of this pilot study indicate that most of the selected quality control assays and protection studies showed no differences between the four pair-wise comparisons of shake flask-grown BCG test preparations and pellicle-grown BCG reference reagents, with limited exceptions. One of the observed differences included the genetic identity of BCG Tokyo, which is known to contain two variants, 172-I (Type I) and 172-II (Type II), differing in Region of Difference 16 [17,18]. While the pellicle-grown reference reagent was largely Type I, the shake flask-grown test preparation was predominantly Type II, possibly because the shake flask growth method offered a selective advantage to the Type II strain. Though the variation in ratio between Type I and Type II in the two preparations did not result in any observed differences in biological activities and protection models in this study, the propagation of different subtypes via the two different manufacturing methods deserves further investigation. Another difference was seen in the delayed hypersensitivity test, where the shake flask-grown BCG Danish test preparation was significantly more potent than its pellicle-grown counterpart.

In terms of protection in animal models, vaccination with the shake flask-grown BCG Russian test preparation resulted in lower lung CFU in the mouse protection study, but higher lung CFU in the guinea pig protection study when compared to the pellicle-grown BCG Russian reference reagent. Finally, the shake flask-grown BCG Danish test preparation resulted in lower spleen CFU than the pellicle-grown BCG Danish reference reagent in the guinea pig protection study. Future studies using more closely matched material (as described below) will determine if this finding is repeatable and whether the differences are attributable to the growth conditions or the lyophilization process. While neither of the protection studies are required BCG release assays, they are important for determining the biological activity of the material and are incorporated here to attempt to more completely characterize the preparations.

These studies represent the first step in comparing BCG strains grown by two different manufacturing methods using assays specifically selected to ensure the quality, safety, and potency of BCG vaccines. In this program BCG strains grown in shake flasks in growth medium containing detergent and stored in glycerol were used as a representation of growth in a fermenter. The fermentation manufacturing process is being considered as a replacement for the traditional pellicle growth method [19], and has already been implemented in the production of new TB vaccine candidates, including the recombinant BCG vaccine candidate, VPM1002, which is currently being tested in the clinic [20]. As the need for a more efficacious TB vaccine persists, the fermentation manufacturing method could easily be applied to novel vaccine candidates, which have the ability to include the new manufacturing process as part of the product application package,

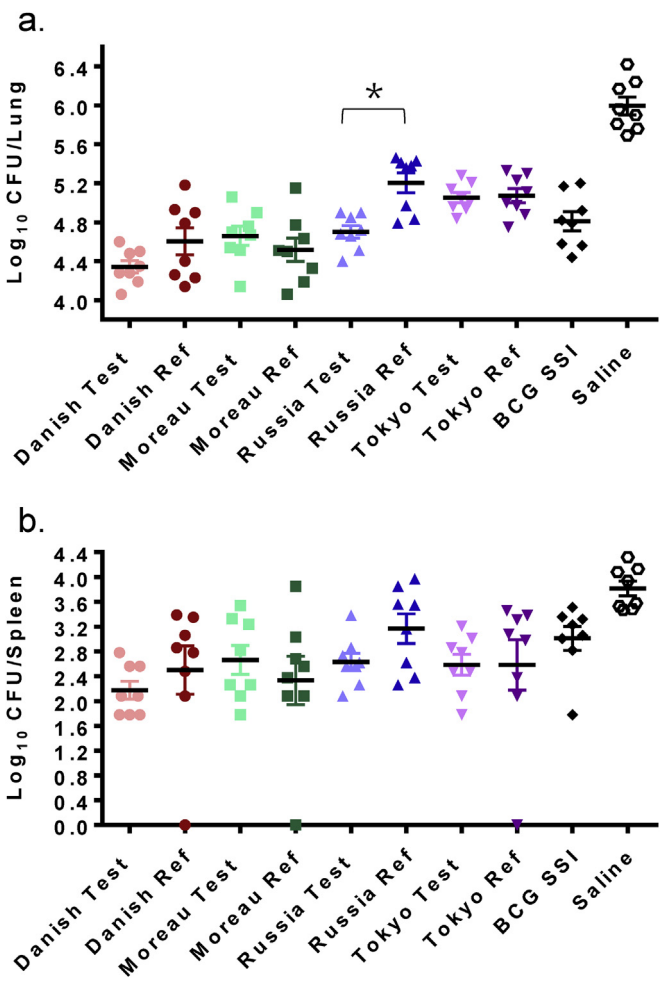

Fig. 4. Protection of Balb/c mice against Mtb H37Rv challenge following immunization with shake flask-grown BCG test preparations or pellicle-grown BCG reference reagents. Bacterial load in lungs (a) and spleen (b) was determined 4 weeks post challenge. BCG SSI and saline were used as assay controls. Individual data points with the mean are shown. Significance was determined for pairwise comparisons only. *$P < 0.01$ by the Mann-Whitney test (lung: $P = 0.0042$).

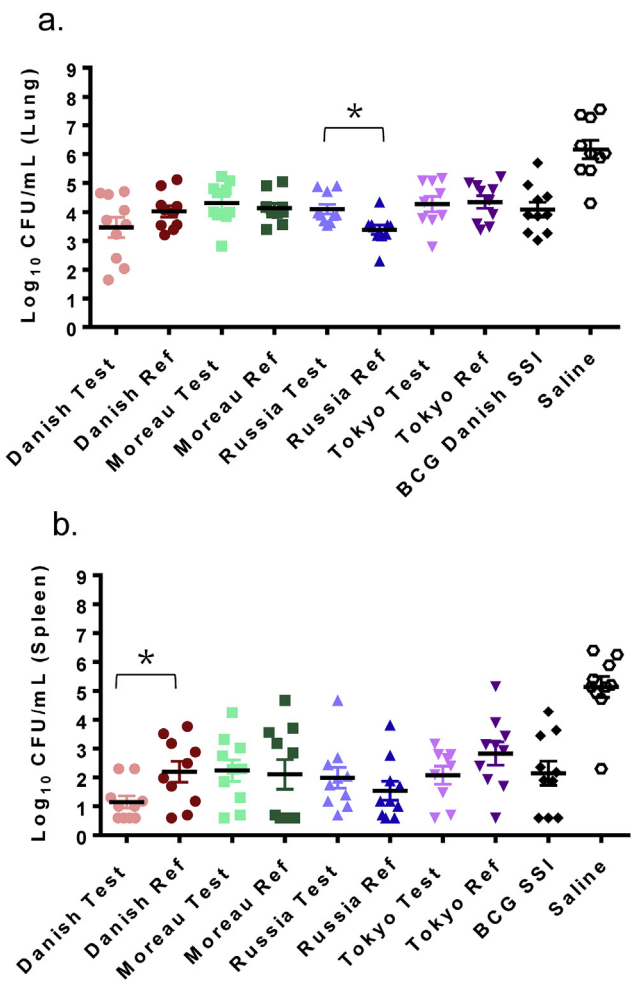

Fig. 5. Protection of female Dunkin Hartley guinea pigs against Mtb H37Rv challenge following immunization with shake flask-grown BCG test preparations or pellicle-grown BCG reference reagents. Bacterial load in lungs (a) and spleen (b) was determined 4 weeks post challenge. BCG SSI and saline were used as assay controls. Individual data points with the mean are shown. Significance was determined for pairwise comparisons only. *$P < 0.05$ by the Mann-Whitney test (lung: $P = 0.0015$; spleen: $P = 0.0261$).

which is not the case for existing licensed BCG.

Using accepted BCG quality control assays, our preliminary studies suggest there are limited observable differences between the stationary, pellicle culturing of BCG compared with culturing in shake flasks. An important next step is to undertake a head-to-head comparison of lyophilized fermenter material with lyophilized pellicle material to ensure the results elucidated in these studies are confirmed. It should also be noted that this pilot study did not account for passage number when comparing growth methods, as the pellicle-grown cultures were passaged less than the shake flask-grown cultures. Future studies should include equal passaging across methods to rule out the possibility that any differences in quality attributes are due to a difference in passage number. In addition, subsequent testing outside of the required assays outlined in the BCG vaccine monograph of the European Pharmacopeia, such as virulence testing using the SCID mouse model, would offer additional support to this data package. Finally, an in depth evaluation of the innate, cellular and humoral immune responses induced by different strains and growth methods may provide valuable insight into the correlate of protection associated with BCG vaccination. Such research investigations could include transcriptomics, intracellular cytokine staining, and functional antibody assays.

The results of this study should encourage more work in this area, as a robust preclinical data package and clinical (non-inferiority) efficacy study may be required to support a changeover in BCG manufacturing methods, a large investment with potential for great impact if it leads to a more scalable system for production of BCG vaccine. The need for BCG vaccine for multiple purposes, including infant vaccinations, clinical trials for new TB vaccines or regimens, preclinical studies, and the treatment of bladder cancer, will continue for the foreseeable future. It is therefore imperative that the worldwide supply is robust and readily available for those in need.

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Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tube.2018.10.013.

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