BCG Vaccine and its use in Mexico

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1 Introduction

Tuberculosis is a high incidence disease worldwide. In 1993, it was declared by the World Health Organization (WHO) as a re-emerging disease due to the increase in the number of cases, often associated with the co-infection with Human Immunodeficiency Virus (HIV) and to the increased number of cases of multidrug-resistant strains which limit therapeutic options to other drugs than isoniazid, rifampicin, ethambutol and streptomycin (Cole, Eisenach, McMurray, & WRJ, 2005).

To date, WHO estimates that one third of the population; approximately 2.33 billion people are infected with M. tuberculosis. That number of infected people is explained, as one person with active TB is able to infect an average of 10 to 15 persons per year and of these 5 to 10% develop the disease. In 2011, WHO estimated 8.7 million new cases of tuberculosis worldwide (equivalent to 128 cases per 100 000 population) and 1 million of deaths (Cole et al., 2005; WHO, 2011).

![Figure 1: Incidence of new cases of tuberculosis published by the WHO in 2012 (WHO, 2011).](image)

The only vaccine currently in use to prevent tuberculosis is the Bacille of Calmette-Guerin (BCG). This vaccine is effective to protect against miliary and meningeal tuberculosis, however, high variability in its efficacy against pulmonary tuberculosis has been reported and the reason which led to the development and evaluation of new vaccines against tuberculosis (WHO, 2004).
2 BCG Vaccine

The first time BCG vaccine was used to prevent tuberculosis took place in 1921. Successful administration of BCG to a child, whose mother had tuberculosis, protected him of acquiring the disease. By 1926, 5,183 babies were vaccinated by oral route applying three doses of BCG, the reports made by Calmette showed a decrease from 25 to 2% in the mortality of children less than one year of age (Hawgood, 1999). In fact, between 1924 and 1929 more than 152,000 children were vaccinated with BCG in France, with no reports of accidents attributed to the vaccine (P. León, 1992). Based on these successful results the liquid BCG vaccine was implemented in the world in order to prevent tuberculosis.

Today, the BCG has been administered to more than three billion people worldwide. Initially BCG was administered orally to infants and subsequently the administration changed to intradermal route, leading to less adverse effects and increased to security, through administration of a lower dose of BCG a better control of the effective dose was allowed worldwide. The last country to use the oral route was Brazil in 1973 (P. Fine, Carneiro, Milstein, & Clements, 1999; WHO, 2004).

Currently the vaccine is produced from different BCG strains, among which are BCG Connaught, Danish, Moreau, Pasteur, Russia and Tokyo accounting the 90% BCG vaccines produced and used worldwide. The recommended dose of BCG vaccine in children is 0.05 mL, although usually are given 0.1 mL containing 200,000 to 3 million of UFC according to BCG used in vaccination (P. Fine et al., 1999; WHO, 2004).

2.1 Development of BCG

The BCG vaccine consists of a live attenuated strain, which was obtained by Albert Calmette and Camille Guérin (Pasteur Institute, Lille, France) from a M. bovis isolate of bovine mastitis (Hawgood, 1999; Oettinger, Jorgensen, Ladefoged, Haslov, & Andersen, 1999). The BCG strain was then attenuated by subcultures on a glycerol-soaked potato slices supplemented with ox bile, a total of 230 subcultures were realized (1908-1921). During this time the loss of virulence of bacillus was documented in several animal models such as cows, guinea pigs, monkeys, etc. Calmette performed the first report of strain attenuation after the fifteenth culture (Bonah, 2005; Hawgood, 1999; Oettinger et al., 1999). From 1921 to 1960 the BCG strain was maintained by continuous culture, implemented as the preservation method of BCG.

Figure 2: Calmette and Camille Guerin, developers of BCG vaccine. Taken from personal collection of Professor Gonzalo Castillo-Rojas.
Due to successful results of BCG vaccination in France, several copies of the BCG culture preserved at the Institute Pasteur de Lille (France) were distributed to various countries of the world (Behr et al., 1999; Oettinger et al., 1999). Historical records showed that the first strain was sent to Russia in 1924 and was called BCG Moscow. Accordingly in this way, each copy of BCG strain derived from the Institute Pasteur was identified as BCG followed by: the country name that received BCG strain laboratory, which produced, or the researcher name who received (examples, BCG Mexico BCG Pasteur and BCG Moreau, respectively). Thus, between 1924 and 1926 thirty-four BCG copies were sent from Pasteur Institute to different countries. Then, next year other 26 more countries received a copy of BCG strain. In the following years the BCG was sent to all countries that requested. Since no formal methodology was achieved, the BCG strains were cultured, preserved and prepared under different conditions. Until now effects of this activity in production path is still preserved. Furthermore, among the BCG derived directly from Pasteur Institute, other places just made a copy from it, for example the BCG Connaught is derived from BCG Frappier, while BCG Glaxo derived from BCG Danish (Behr et al., 1999; Hawgood, 1999; Oettinger et al., 1999).

Over time differences in morphological characteristics (macroscopic and microscopic) and protective efficacy were documented among BCGs, and attributed to the different conservation methods used for preservation of this strain (Aronson & Schneider, 1950; Dubos & Pierce, 1956). Since the BCG strains were preserved by subcultures until 1960, year that begins to be used the freeze dry as preservation method and, until then the production method changed to “seed lot”, which leads to production of lyophilized BCG vaccine. To date, it is clear that differences exist among BCG strains, which generated the need to characterize them at different levels. On the following we attempt to describe some documented differences between BCG.

### 2.2 Immunity and Protective Efficacy

The study of the effectiveness of BCG is divided into two prominent groups: a) the protection that BCG confers against miliary and meningeal tuberculosis in children and b) the protection that BCG confers against pulmonary tuberculosis in adults.

In respect to the first group, there are consistent results showing that BCG protects against miliary and meningeal tuberculosis in children, with a protective efficacy of about 80% (Andersen & Doherty, 2005; Trunz, Fine, & Dye, 2006). However, the protection that BCG confers against pulmonary tuberculosis in adults is highly variable with an efficacy between 0 and 80%. In general terms, the protective efficacy of BCG varies between 0 to 100% depending on host and BCG factors such as the BCG used, the age of BCG application and the geographic region in which the study is conducted. For example, the BCG Copenhagen showed a 77% protection in children from England; although the same BCG vaccine applied to general population in Colombia showed a protection of 0% (P. Fine et al., 1999; P. E. Fine, 1988). A meta-analysis published in 1994 based on 16 studies found that BCG reduces the risk of developing pulmonary tuberculosis in 50% of cases (Colditz et al., 1994).

The ultimate reasons for this variation are not yet clear, however, several factors could be responsible of affecting BCG vaccine efficacy. Among others related to vaccine differences, already mentioned, are the exposure to environmental mycobacteria, genetic profile of the population and differences in the virulence of *M. tuberculosis* strains (P. Fine et al., 1999; WHO, 2004).
2.3 Differences between BCG

The first documented differences were morphological changes as size, shape and texture of the colonies on solid medium. By 1950 Deinse Van et al described that the number of subcultures on Sauton medium and the time between each one caused changes in the colonial morphology and growth time of BCGs (Van Deinse & Senechal, 1950). The report of these differences led to an extensive study of the phenotypic characteristics of BCGs and therefore the description of a greater number of differences (Oettinger et al., 1999).

Subsequent studies revealed differences in antigen production among BCGs, such as MPB64 and MPB70. These antigens MPB64 and MPB70 have been described as immunogenic proteins in mycobacteria. BCGs obtained from Pasteur Institute before of 1927 secrete these antigens, whereas BCGs distributed after 1927 are unable to synthesize these antigens (Charlet et al., 2005; Oettinger et al., 1999). This feature classified a BCG into two groups as "early" and "late", regarded as "early" the BCG strains capable of secrete both antigens.

Later on, it was found that BCGs differ in their capability to produce mycolic acids. These compounds are in the cell wall of mycobacteria and are of three types: alpha-mycolic acids, methoxy-mycolic acids and keto-mycolic acids. BCGs obtained after 1931 from Pasteur Institute are unable to produce methoxy-mycolic acids due to a point mutation in mma3 gene (Behr, Schroeder, Brinkman, Slayden, & Barry, 2000; Belley et al., 2004).

Besides, other antigenic changes, proteins ESAT-6 and CFP-10, two of the most studied antigens from mycobacteria. In BCG are within the RD1 region, which is lost in all BCG strains. Importantly this antigens can however, differentiate the BCG from *M. tuberculosis* and *M. bovis* (Liu, Tran, Leung, Alexander, & Zhu, 2009)

An extensive characterization of the BCG has been done to find out in which extent variability in strain protection happens to work against tuberculosis.

In this context, genomics allowed to study thoroughly the differences among BCGs. One of the first tests used to BCG comparison was done by the pulsed field gel electrophoresis (PFGE), analysis of repeating units of mycobacteria (MIRUS) and variable number repeat sequences (VNRTs), which showed different profiles among BCGs, however, at this scale of comparison, it was not possible to find a clear difference among them that is clearly associated with the efficacy to induce protection against tuberculosis (Frothingham, 1995; Varnerot, Clement, Gheorghiu, & Vincent-Levy-Frebault, 1992). Although, a genetic marker widely used was discovered, the presence of the insertion sequence IS6110, in the BCG derived from Pasteur Institute before 1927 which has two copies and BCG obtained after this date it is availed only as one copy BCG strain (Cole et al., 2005).

The most important works in this field were performed by G. Mahairas, M. Behr and R. Brosch (Behr et al., 1999; Brosch et al., 2007; Brosch et al., 2002; Mahairas, Sabo, Hickey, Singh, & Stover, 1996). In these studies is described the presence of genetic deletions in BCG in comparison with *M. tuberculosis* and *M. bovis*, which were called regions of difference (RDs).

Mahairas *et al* determined the existence of absent RDs in BCGs but it’s present in *M. tuberculosis* H37Rv (Mahairas et al., 1996). Moreover, Brosch *et al* described new RD regions, using bacterial artificial chromosomes (BACs), which are present or absent depending on the BCG analyzed (Brosch et al., 2002). This feature allowed distinguishing these strains from *M. tuberculosis* and development a polymerase chain reaction (PCR) for BCG identification. By the late 90’s, Behr performed a microarray with 13 BCGs that led him to identify new RD regions, also found that the presence of these regions correlates
with historical data from Pasteur Institute (Behr et al., 1999). This finding led to the first genealogy of BCG strains construction.

Nowadays, more than 15 RD regions have been described between BCG and *M. bovis*, some of which are responsible for the phenotypic differences observed between them (Liu et al., 2009). For example, the RD1 deletion removes 9 genes (PE35, PPE68, ESAT-6, CFP10) and is useful to distinguish the BCGs of *M. bovis*. Interestingly, it was found that deletion of this region in *M. tuberculosis* induces its attenuation, however, the complementation of BCG with the genes encoded by RD1 does not restore the virulence of BCGs (Lewis et al., 2003; Pym, Brodin, Brosch, Huerre, & Cole, 2002). On the other hand, Kozak et al. recently reported that the presence of the RD2, a region absent in the late BCGs, is associated with immunogenicity induced by the BCGs (Kozak & Behr, 2010). In spite of these findings, these deletions do not explain all phenotypic differences observed among BCGs, suggesting that other regions are involved in the attenuation of BCG strain.

In addition to RD regions, also the presence of two tandem duplications (DU) in the BCG has been described, called DU1 and DU2, of which DU1 is only in BCG Pasteur. The presence of these regions appear as genes duplication in BCGs, however, the functional implications of these duplications are not known (Brosch et al., 2000; Brosch et al., 2007; Brosch et al., 2002).

Was in 2007 when the first genome of a BCG strain was released, corresponding to the BCG Pasteur 1173P2, whose genome is constituted of 4 374 522bp. The BCG Pasteur genome was approximately 30Kb larger than *M. bovis* genome and once its genome sequence was revealed and subsequent analysed, it allowed a genealogy refined of BCG vaccines to be constructed incorporating, the RD and DU2 regions (Figure 3) as markers. Along the RD2 presents different sizes among BCGs, a classification into four groups, DU2 type I-IV was a possible match (Brosch et al., 2007).

Recently, the complete genome sequence of BCG Tokyo 172 and BCG Moreau were published, which had a size of 4 371 711bp and 4 340 116 bp, respectively. Also, the draft genome sequences of four other BCG strains (China, Danish 1331, Russia, Tice) were reported on 2011, each one of approximately 4.27 Mb (Gomes et al., 2011; Pan et al., 2011; Seki et al., 2009).

Comparison between BCG Tokyo and Pasteur sequences showed the presence of mutations in BCG Tokyo, including insertions and deletions, not described previously (Seki et al., 2009). The analysis of BCG Pasteur and Tokyo sequences and its comparison with the sequences of *M. tuberculosis* and *M. bovis* also led to the identification of single nucleotide polymorphisms (SNPs), both unique and shared between the BCGs (Brosch et al., 2007; Garcia Pelayo et al., 2009; Seki et al., 2009). Some of the described SNPs have functional inferences on the genes they affect; an example is the SNP in the gene *mma3* that is responsible for the loss production of the methoxy mycolic acids in the late BCGs (Behr et al., 2000). In fact, in the last years the study of SNPs has taken force due to the functional implications that could be attained, especially if it is considered that the polymorphisms described previously (deletions and duplications) not completely explain the over-attenuation observed in the BCGs.

In summary, the study of the BCGs at genomic level allowed establishing specific differences among these strains, and in some cases to identify genomic profiles used to develop identification methods for BCG strains. Moreover, the genetic analysis of BCGs led to identify specific relations between the presence of mutations and its attenuation.
With all this knowledge the obvious question is if the genomic differences among BCGs are the cause of changes in the protein expression profile. The proteomic profiles described for the BCGs included the BCG Chicago, Copenhagen, Pasteur, Danish, Phipps and Moreau. The analysis showed differences (presence / absence) in the protein expression from cellular fraction, culture filtrate and in the membrane proteins obtained under standard culture conditions (Berredo-Pinho et al., 2011; Jungblut et al., 1999; Rodriguez-Alvarez et al., 2010).

Recently, Rodríguez-Alvarez et al realized the comparison between the BCG Phipps and Danish proteomes. In this study, they found over- expressed the proteins alkyl hydroperoxide reductase C (AhpC), the culture filtrate protein 17 (CFP17) and the chaperones GroEL1 and GroEL2 in BCG Phipps; whereas in BCG Danish were over- expressed the proteins GroEL2, TB39.8, the protein of 35KDa and one probable alcohol dehydrogenase. All these proteins are involved in the virulence, detoxification and different adaptation of mycobacteria. (Rodriguez-Alvarez et al., 2009)

Berredo-Pinho et al, who perform a comparison of proteomic profiles between BCG Moreau and Pasteur, obtained similar results. They, also described differences in the proteins of culture filtrate ex-
expression, some of them described in the literature as immunogenic antigens or potential targets of new candidates vaccine against TB (Berredo-Pinho et al., 2011).

The evidences mentioned above highlights the need of continuing BCG strains characterization to understand the mechanisms of attenuation, and the information generated to be used for new vaccines rational design. At present, the WHO has a working group that realize expectative in research of BCG characterization. Besides, these group also gives recommendations in using standard methods to persuade this aim, such as the variable number of repetitions in tandem determination (VNRT), the study of the RD and DU regions, tests of potency and efficacy of the BCG vaccines, (Walker et al., 2010).

2.4 New vaccines against tuberculosis

The BCG vaccine has variable efficacy against pulmonary tuberculosis in adults. This variability together with the increase in the number of tuberculosis cases and the risk of BCG administration to HIV positive subjects generated the need to develop alternatives to prevent this disease as mandatory.

The development of new vaccines against tuberculosis began in the 90’s, arriving to a plethora of possibilities. Although in general, the objective of designing new vaccines against tuberculosis split into two groups: vaccines that are proposed to replace the BCG and vaccines that could be used as a booster. In the first group there has been proposed the use of recombinant BCG, *M. tuberculosis* attenuated and *M. vaccae* as examples. Whereas, in the second group are best suited the subunit vaccines. Also, the development of therapeutic vaccines has been considered as RUTI (Brennan & Thole, 2012; McShane, 2011; Ottenhoff & Kaufmann, 2012).

These vaccines must be tested first in in vitro studies and experimental animal models, to appear as possible candidates those, which has equal or greater protective efficacy against tuberculosis than the efficacy that is conferred by the BCG vaccination. However, tuberculosis vaccine evaluation is a difficult task since no biomarker exist that can be directly correlated with protection, even neither to a human challenge model. This leads to test the efficacy of the candidates in clinical trials, the first one was conducted in early 2002 (Brennan & Thole, 2012; Ottenhoff & Kaufmann, 2012). The design of clinical studies face several practical order challenges like: the selection of study population, and determine tuberculosis incidence, to get the necessary budget to carry out the study among others. In fact, between 2005 and 2010 more than $ 600 million of dollars were invested, leading to only 15 vaccines candidates tested in over 50 clinical studies (Small, 2012).

Although early results of clinical studies will be available in the next few years, a long path remains ahead in the development of new vaccines against tuberculosis. In fact, a recent published a project raised key points to follow in the search for new vaccines against tuberculosis including creativity in research and development, correlation of immunity and biomarkers in vaccines against tuberculosis, harmonization and cooperation in clinical studies, rational selection of new vaccine candidates, and the critical need to promote and funding (Brennan & Thole, 2012). It is important to mention that commitment has appeared as a result of collaboration from different public and private institutions, governmental and non-governmental organizations, pharmaceutical companies and several groups of researchers around the world with new proposals against tuberculosis in conferences and meetings.
3 Use of BCG in Mexico

Fernando Ocaranza introduced the BCG vaccine in Mexico in 1925 to prevent tuberculosis. At this time Mexico scored a high number of tuberculosis cases, just below from smallpox and typhus diseases, so the use of BCG was justified to combat this disease (Instituto-Nacional-de-Higiene, 1995; P. León, 1992). However, it was not until 1931 that the BCG vaccine was produced for first time at the Hygiene Institute under doctor's request. Initially this BCG vaccine was applied to child whose mothers had tuberculosis, at that time, the BCG immunization schedule consisted of three doses to newborn in a period of 10 days (Higiene, 1931).

The BCG vaccination continued under this scheme for the next 15 years, but it not until 1949, that the BCG vaccine was produced on a large scale to use in Mexican population. By this time BCG vaccine was given to children during the first 10 days of life and to negative PPD adults. The BCG vaccine started by oral route administration and then changed to an intradermal route because the former administration route could lead to presence of cervical lymphadenopathy or BCGitis, which occurs when disseminated BCG infection happen, in vaccinated children. From February 1949 to June 1950 a total of 22 937 children younger than one year old, 7 669 children older than one year of age and young adults were vaccinated with the BCG México (Escobar, 1992; P. León, 1963; P. León & Jiménez, 1950; P. León, 1992).

During the following years quite a lot argumented discussion by doctors and the general community took place about to go on using BCG in Mexico or not, leading to under achievement in the vaccination coverage initially estimated. In both cases the argument against BCG vaccination was that it could lead to sick or die of tuberculosis in vaccinated children, because “this vaccine was not consider safe”. In Mexico and around the world this issue was quite a lot discussed due to results in clinical trials of BCG vaccines and in animal models obtained, (Fernández de Castro, 1976; P. León, 1992). A conclusion began to be generated as information on the frequency of serious complications attributable to BCG was compared with other vaccine used at that time. It was not a surprise to discover and and clearly demonstrate that BCG has the lowest incidence of severe complications in comparison to vaccines against measles, pertussis, yellow fever and smallpox.

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**Figure 4**: Stamps issued in Mexico with reference to the campaigns of TB in the country. Taken from Dr. Gonzalo Castillo-Rojas personal collection.
Since then, the use of BCG took a new impulse and in 1961 began to be applied to all children under 15 years. In fact, it was by 1969 that direct vaccination method was established, as the immunization was carried out without previously performing PPD test. Thus, between 1973 and 1974 about 17 million BCG doses were applied, reaching a 70% coverage (Fernández d Castro, 1976). This vaccination program impacted on incidence reduction of tuberculosis in Mexico.

BCG vaccination against tuberculosis continued to perform in the country; with variable vaccination coverage in the 80’s (15-80%), and for the early 90’s vaccination coverage increased to 90% reaching 99% in 1996, and levels remain so until today. Currently, NOM-0.36-2002-SSA2 from Mexico stipulates that BCG vaccine should be applied to children during the first year of life by intradermal route, the authorized BCGs are the following: BCG Pasteur 1173P2, BCG Montreal, BCG Danish 1331, BCG Glaxo 1077 and BCG Tokyo 172.

3.1 BCG Vaccine Production

Production of liquid BCG vaccine in Mexico began in 1931 from BCG strain sent directly by Calmette, Institut Pasteur. Now is known that a number of different shipments of BCG were provided to Mexico, this fact is supported by letters of request to Institute Pasteur by Alfonso Reyes, dated July 1 of 1926, February 12 and March 7 of 1927. Accordingly in those years studies began at the Institute of Hygiene to perform the growth of the BCG strain in Sauton medium and test different preservation methods (Orduna et al., 2011).

In year 1929 the BCG Laboratory facilities at the Institute of Hygiene were inaugurated, and the BCG assays and continued production took place. However, until 1931 when the vaccine began its production on a small scale, and under doctor's request production, under the same scheme also supplied demand for use in animals, mainly for cattle (Higiene, 1931).

The same production method continued until 1949, year that expands the National Institute of Hygiene and BCG laboratory production to supply all BCG doses necessary for Mexico and some Latin American countries. In this period the BCG vaccine was produced from the BCG shipped directly from the Institute Pasteur in Paris with the appointment number of 870 Series I (called BCG Mexico), which was preserved by duplicate by continuous cultures on glycerinated potato medium supplemented with ox bile, with intervals of two weeks (Escobar, 1992; P. León, 1963; P. León & Jiménez, 1950).

In 1963 a special committee for Biological Products Control from a WHO division approved the use of the BCG vaccine produced in Mexico. In comparative studies with Danish BCG (reference strain) it was demonstrated that BCG Mexico produced in the Mexico had a power and quality comparable to Danish strain. This achievement led to consolidate nationwide distribution, as well to initiate foreign distribution to several countries in Central America and South America. During this time, more than 15 million children were vaccinated with the BCG produced in Mexico (P. León, 1963).

The production of BCG Mexico strain continued until 1970, year in which the production method was changed to “seed lot” (lyophilized BCG). Then after BCG vaccine production from BCG Danish 1331 strain began in Mexico. The BCG strain change is explained because the Danish BCG had already standardized methodology for lyophilisation, although the BCG Mexico was less attenuated than BCG Danish (Fernández d Castro, 1976).

The production by the seed lot method requires that lyophilized seed original must be grown over 12 continuous cultures (actually, never more than 8 crops) to prevent a possible BCG genomic variation, and BCG strain replacement by this phenomenon, prevents different properties acquisition in addition to
maintain a consistent quality of the BCG vaccine. Thus began the production of BCG Danish vaccine with an international quality, with an advantage over the liquid vaccine because of higher conservation in adverse environmental conditions (Fernández d Castro, 1976).

In 1998 the BCG Danish vaccine production in Mexico ended. Since that year BCG is imported from any commercial BCG vaccine available production retail, as example BCG Danish, BCG Tokyo and BCG produced in India, were distributed in Mexico regardless of their quality, potency and efficacy. This situation originated Mexico to use a wide variety of BCG vaccines and made protective efficacy uncertain. There are also reports, uncommon, of a lack of supply of BCG vaccine in health systems, resulting in the absence of vaccination for up to six months. In addition, to previous, importing of BCG vaccine also leads to an increase in the cost of vaccination, even when trained staff in the preparation of this vaccine is still an active workforce. All this pieces of information led to discuss the convenience of producing BCG Mexico back again in the country since many studies have supported its characterization at diverse levels.

Based on studies and history of vaccination with BCG Mexico in at least 20-year window in our country, research groups did the BCG Mexico genomic characterization. A very useful tool to find similarities and differences with other BCG such as Pasteur and Tokyo and future construction of second-generation vaccines based against tuberculosis.

3.2 Characterization of BCG Mexico

As described in the previous section BCG Mexico was used to produce the BCG vaccine in Mexico for a period of more than 20 years, however, this strain has been characterized by us since 2000 where began studies on acquired immune response in mouse model of progressive pulmonary tuberculosis, then on the characterization of BCG Mexico, which initially comprised determining their RDs profile, its complete genome and proteome (Orduna et al., 2011). Nowadays, the studies continue with the innate immune response characterization are going on.

The characterization of BCG Mexico at genomic level showed that this BCG is located in DU2 type IV group (Figure 3). The RDs profile determination by PCR showed that BCG Mexico conserved RD8, RD14, RD16 and RD D/G regions and lost RD1, RD2, N-RD18 regions and a copy of insertion sequence IS6110. A profile indistinguishable from the profile described to BCG Phipps and Tice.

In order to have a full genetic characterization of BCG México, the complete genome of BCG Mexico was sequenced. The result showed that BCG Mexico genome contains 3904 CDS, 3 genes coding for rRNA and 45 tRNA genes, additionally 29 pseudogenes were identified (Figure 5). These results showed that BCG Mexico genome contains fewer genes annotated (3904) than BCGs Pasteur (3954) and Tokyo (4033), this variation is due to different numbers of RD regions in the strains and differences in annotation of hypothetical proteins.

Comparison of the sequence obtained for BCG Mexico with the BCG Pasteur and Tokyo sequences identified three regions absent in BCG Mexico, which were designated RDMex01 (53bp) RDMex02 (655bp) and RDMex03 (2 847bp). Interestingly, analysis of these regions in nine BCGs showed that RDMex03 and RDMex02 are absent only in BCG Mexico, which can be considered as molecular markers of BCG Mexico (Orduna et al., 2011).
Figure 5: Circular map of the BCG Mexico chromosome. The scale is in bases and is shown in the black outer circle. The dark blue circle shows forward-strand CDS, and the light blue circle shows reverse-strand CDS. The next two circles moving inward show pseudogenes (green) and difference regions (red). The innermost circle represents the G+C content (Orduna et al., 2011).

RDMex01 is an intergenic deletion located between the BCG0767 (rpsN1) and BCG0768 (rpsH) genes, which encode two subunits of the 30S ribosomal protein (Table 1). The biological effect of this deletion is unknown. RDMex02 is associated with deletion of 218 aa from BCG3889 (fadD23), affecting a conserved region of the protein that includes two transmembrane domains (Table 1). This gene encodes a probable fatty-acyl CoA ligase involved in lipid degradation. Lynett et al. have reported that this protein is involved in sulpholipid production and that disruption of the gene results in increased association between bacteria and macrophages (Lynett & Stokes, 2007). Additionally, Molina et al. found that BCG Mexico associates more strongly with macrophages (THP-1) compared to BCG Danish, BCG Moreau, BCG Phipps and BCG Tokyo172 (Molina-Olvera, 2010). This is interesting because this association to macrophages can result in greater internalization of strain.

Finally, RDMex03 was the largest deletion found in the BCG Mexico genome (Table 1). It affected four genes: three genes encoding hypothetical proteins (BCG3923, BCG3924 and BCG3926) and another gene encoding a putative transcriptional regulator [BCG3925c (whiB6)] belonging to the WhiB protein family (1-7). This family has been proposed to form part of a new redox system in M. tuberculosis (Alam, Garg, & Agrawal, 2009). Interestingly, this deletion is situated in the extended RD1 region.
Evidence suggesting that these proteins are transcription factors involved in regulating cell processes as cell division, pathogenesis and antibiotic resistance. The absence of this gene in strain can cause a differential response of BCG Mexico to oxidative stress compared with other BCGs, which may be involved in the survival of the strain within the macrophage.

As well as being considered as molecular markers of strain, the new RDs described in BCG Mexico 1931 may contribute to understanding of the phenotypic differences between BCG Mexico and other BCG strains.

<table>
<thead>
<tr>
<th>Deleted sequence</th>
<th>Location</th>
<th>Length (bp)</th>
<th>Affected genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>RDMex01</td>
<td>844357…844410</td>
<td>53</td>
<td>Intergenic region between rpsN1 and rpsH</td>
</tr>
<tr>
<td>RDMex02 (Δfad23)</td>
<td>4272815…4273470</td>
<td>655</td>
<td>fadD23</td>
</tr>
<tr>
<td>RDMex03 (ΔRv3860-64)</td>
<td>4308821…4311668</td>
<td>2847</td>
<td>BCG3923, BCG3924, whiB6, BCG3926</td>
</tr>
</tbody>
</table>

Table 1: New RD regions identified in BCG Mexico 1931. 1 Locations and names of affected genes are given according to the BCG Pasteur 1173P2 genome; 2 also deleted in BCG Danish 1331 (Orduna et al., 2011).

The comparison between BCG Mexico sequence and BCG Pasteur and Tokyo sequences showed the presence of SNPs, a total of 37 SNPs representing nonsynonymous mutations (nsSNPs) were identified, leading to amino acid substitutions. Four SNPs in this group were specific to BCG Mexico 1931 [BCGMEX_0506c, BCGMEX_1957, BCGMEX_2390 and BCG3741 (ponA2)]. On the basis of functional classes, the genes containing nsSNPs encoded hypothetical proteins (19%), proteins involved in intermediary metabolism and respiration (16%), proteins related to lipid metabolism (16%) and PE/PPE family proteins (16%) (Orduna et al., 2011).

The analysis found SNPs within BCG0510c (pcaA), BCG0532 (regX3), BCG0692c (mma3), BCG0484c (sigK) and BCG3734 genes. The SNPs in the last three genes have been described in previous studies (Garcia Pelayo et al., 2009; Seki et al., 2009). The SNP found in BCG0692c (mma3) causes an amino acid change with the loss of methoxymycolates in BCG strains obtained from the Pasteur Institute after 1927. This result was consistent with the findings of Hayashi et al., who described the absence of these acids in BCG Mexico 1931 (Seki et al., 2009). An SNP in the start codon of BCG0484c (sigK) is responsible for low expression of MPB70 and MPB83 in late BCG strains, including BCG Mexico (Charlet et al., 2005). Moreover, mutations in BCG3734, a CRP homologue global regulator, have been described as specific to BCG and are responsible for increased binding of CRP to its target DNA (Bai, Gazdik, Schaak, & McDonough, 2007). Mutations in Rv0491 (regX3) and Rv0470c (pcaA) have been implicated in the virulence of M. tuberculosis. The pcaA gene encodes a mycolic acid cyclopropane synthetase and is important for growth, persistence in macrophages and proinflammatory activity (Glickman,
Cox, & Jacobs, 2000; Rao, Fujiwara, Porcelli, & Glickman, 2005). Additionally, regX3 is part of a two-component system regulated by Pi (SenX3-RegX3) that is involved in the virulence of M. tuberculosis (Glover, Kriakov, Garforth, Baughn, & Jacobs, 2007; Parish, Smith, Roberts, Betts, & Stoker, 2003).

Interestingly, a specific nsSNP from BCG Mexico causes an amino acid change in BCG3741 (ponA2). Mutations in this gene have been associated with increased sensitivity to heat shock (24 h at 45°C) and exposure to H2O2 compared to wild-type M. tuberculosis. Additionally, a ponA2 mutant was found to exhibit lower survival in mice compared to wild-type M. tuberculosis (Vandal et al., 2009).

In the analysis also were identified six SNPs in PE_PGRS4, PPE22, PE_PGRS41, PPE50 and PE_PGRS43b genes. These genes encode PE/PPE family proteins, which may play a role in the evasion of host immune responses, possibly via antigenic variation of mycobacteria. In previous studies, it has been shown that the PPE22 protein elicits B cell responses, while PPE50 is required for mycobacterial growth in vitro (Sampson, 2011; Sassetti, Boyd, & Rubin, 2003). Furthermore, it was determined that PE_PGRS54 (6,285 bp) and PPE_PGRS55 (5,433 bp) correspond to a longer product in BCG Mexico compared with homologous sequences only for BCG Tokyo (6,153 and 5,088 bp, respectively). These results are consistent with data previously described (Seki et al., 2009). Importantly, the functional implications of these size variations remain unknown.

Moreover, proteome of BCG Mexico was determined as part of characterization: in the BCG proteome were identified 812 proteins (Figure 6). On the other hand, an immunoproteome performed with sera from PPD +, PPD- and active tuberculosis subjects led to identification of 39 reactive spots (Figure 6B). The largest numbers of reactive spots were obtained when using serum from subjects with active TB (16) or positive tuberculin skin test results (PPD+) (14); 12 of these spots were unique to each serum type (Figure 6B). This result indicates high variability in the proteins recognised by each type of serum. From this immunoproteome, a total of 37 proteins were identified by sequencing, the majority of which (17; 47%) corresponded to intermediary metabolism and respiration proteins (Figure 6C). Among the identified proteins, some have been previously described as virulence proteins in different strains of M. tuberculosis: phosphoenolpyruvate carboxykinase (pckA), isocitrate lyase (icl), 3-oxoacyl synthase II (kasA), groEL, TB27.3, the 85A and 85C antigens, alkyl-hydroperoxide reductase (ahpC) and heat shock protein HspX. The proteins phosphoenolpyruvate carboxykinase, isocitrate lyase, 3-oxoacyl synthase II and AhpC were described as antigenic proteins for the first time in this report. The study of these proteins may be useful for the development of new diagnostic reagents and to know their role during infection.

In summary, the characterization of the BCG Mexico led to identification of the strain-specific polymorphisms, which have an impact on phenotypic characteristics of BCG Mexico.

4 Future Challenges

Just half the way to attain a consistent knowledge of one of the most paradigmatic vaccines ever, there’s a lot to be learned about BCG, in the years for coming, and as first steps in characterization are in progress, the mechanisms involved in its attenuation still claims to be elucidated. This knowledge would be useful for development of new vaccines against tuberculosis. In this field there are still many overhead obstacles, these can be overcome with the cooperation of all those involved in this field.
Figure 6: Immunogenic proteins identified in BCG Mexico, a total of 37 immunogenic proteins were identified. (A) Representative proteome of BCG Mexico. Spots circled in red, green, dark blue and light blue represent proteins reactive to sera from subjects with active pulmonary tuberculosis, NTM mycobacteriosis, PPD+ and PPD-, respectively, while the red, orange and green squares represent proteins shared between TB-MNT, TB-PPD+ and TB-PPD- sera, respectively. (B) Reactive proteins by type of serum. (C) Immunogenic proteins classified by functional category (Orduna et al., 2011).
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