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DEVELOPMENT OF A RECOMBINANT COMPONENT FOR ACELLULAR PERTUSSIS VACCINE DTaP

Biodev Student: Thaísa Scheuer

Supervisor: Carlos Ricardo Soccol, PhD

Co-Supervisor: Vanete Thomaz Soccol, PhD

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THAÍSA SCHEUER

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Resumo

A vacina tríplice bacteriana, contra difteria, tétano e pertussis, possui diversos efeitos adversos, principalmente relacionados ao componente pertussis. Este trabalho pretendeu desenvolver um fração deste componente, possibilitando a produção de uma vacina acelular (DTPa), mais segura. Foi utilizada a tecnologia recombinante através da qual uma porção do gene que codifica a toxina pertussis foi clonada por Reação em Cadeia da Polimerase (PCR) e expressa em *Escherichia coli*. As condições de indução otimizadas (0,1 mM IPTG, 3 horas, 37°C) levaram a um rendimento de 12 $mg.L^{-1}$ da proteína recombinante após a purificação. A identidade da proteína foi confirmada por Western blotting e espectrometria de massas. Desta forma, o presente trabalho desenvolveu uma metodologia adequada visando a produção industrial de DTPa no Brasil.

Palavras-chave: DTPa, *Bordetella pertussis*, vacinas recombinants, coqueluche

Abstract

Triple bacterial vaccine, against diphtheria, tetanus and pertussis has several adverse effects, mainly related to the pertussis component. This work intended to develop a fraction of this component, enabling the production of an acellular vaccine (DTaP), safer. It was used the recombinant technology through which a portion of the gene encoding pertussis toxin was cloned by Polymerase Chain Reaction (PCR) and expressed into *Escherichia coli*. The optimized induction conditions (0.1 mM IPTG, 3 hours, 37°C) led to a yield of 12 $mg.L^{-1}$ of the recombinant protein after purification. Protein identity was confirmed by Western blotting and mass spectrometry. So, present work has developed a suitable methodology aiming industrial production of DTaP in Brazil.

Keywords: DTPa, *Bordetella pertussis*, recombinant vaccines, whooping cough

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Arg - arginine

BCG – *Bacillus Calmette-Guérin*

bp – base pairs

CV – column volume

DAB - 3,3′-Diaminobenzidine tetrahydrochloride

DNA - deoxyribonucleic acid

DT – diphtheria toxoid

DTaP – diphtheria, tetanus and acellular pertussis vaccine

DTP – diphtheria, tetanus and pertussis vaccine

FHA - Filamentous hemagglutinin

Glu – glutamic acid

IPTG - Isopropyl β-D-1-thiogalactopyranoside

Kb – kilobase

kDa - kilodalton

L - liter

LB – Luria-Bertani medium

Lf – limit of flocculation

LPB – Laboratory of Bacterial Products for Human Use

LPS - lipopolysaccharide

min - minute

mL - mililiter

mM - milimolar

MW – molecular weight

ng - nanogram

nm - nanometer

OD – optical density

PBS – phosphate-buffered saline

PCR – polymerase chain reaction

PMT – tetanus monomeric protein

MALDI-TOF - Matrix-assisted laser desorption/ionization – time of flight

PNI – National immunization program

PT – pertussis toxin

s - seconds

SDS-PAGE – polyacrylamide gel electrophoresis with sodium dodecyl sulphate

TECPAR – Technology Institute of Parana

TT – tetanus toxoid

UFPel – Federal University of Pelotas

UFPR – Federal University of Parana

V - volts

xg – gravity

WHO – World Health Organization

μ - specific growth rate

μg - micrograms

μL – microliters

1 PRESENTATION

The Federal University of Paraná (UFPR) is the oldest educational institution conceived as a university in Brazil. It was originally founded in 1912 under the name University of Paraná. Today, demonstrates excellence in its undergraduate, specialization, master's and doctoral degrees that are guided by the principle of teaching, research and extension.

The Department of Bioprocess Engineering and Biotechnology, belonging to this university, is composed of undergraduate and postgraduate. This latter including three main areas: agribusiness and biofuels, agrifood biotechnology and human and animal health. Within this last area of concentration, this study was conducted. In addition, the course values the training of professionals with skills and expertise to participate actively and interdisciplinary in the activities of the industrial market of biotechnology and the development of new products. Aiming at this objective, this work was developed in partnership with the Technology Institute of Parana (TECPAR).

This Institute is a public corporation created in 1940 and linked to the Ministry of Science, Technology and Higher Education of Paraná and is active in research, technological development and innovation, offering technologic solutions to businesses and biological, biopharmaceuticals and medicines to the public sector. TECPAR consists of numerous laboratories, including the Laboratory of Bacterial Products for Human Use (LPB). This laboratory has been responsible over many years for the production of the Tetanus Monomeric Protein (PMT), an raw material used as carrier protein that is conjugated to the polysaccharide found in the extracellular capsule of *Haemophilus influenzae* type B, to obtain the Hib vaccine (against one type of bacterial meningitis). Currently, this protein/polysaccharide conjugate is used in the composition of the quadrivalent vaccine, which is part of mandatory childhood immunization schedule, allowing that with one single dose children are protected against tetanus, diphtheria, pertussis and meningitis.

Among the branches that the institute works, biopharmaceuticals production is one of the research areas that demand improvement, especially regarding the development of new products and improvement and creation of new methodologies applicable to production lines. Therefore, this project was developed with the purpose of bringing the Institute, Federal University of Paraná and Brazil technological autonomy earning in the development of vaccines that are already used in other countries.

2 BIBLIOGRAPHIC REVIEW

2.1 Bordetella pertussis

2.1.1 Disease and epidemiology

Pertussis, also known as whooping cough, is a disease of the respiratory tract that affects humans of all ages but has the great morbidity and mortality in young children especially in babies less than 1 year of age. This disease occurs due to infection by *Bordetella pertussis*, which releases a number of virulence factors, including pertussis toxin (PT), filamentous hemagglutinin (FHA), pertactin and fimbriae. (LEE et al., 2002; CDC, 2012). The main symptoms of this disease are violent coughing which often makes it hard to breathe. (CDC, 2012)

This disease is endemic and presents cycles of three to five years, and, nowadays ranks fifth among the vaccine-preventable diseases in children under five. (WHO, 2011). Since 2010, it has been observed a global trend of increasing number of cases of pertussis, with reports in California and elsewhere in the Americas.

Even with high vaccine coverage - vaccination was responsible to avoid 37 million cases of illness and 587,000 deaths in 2002 (WHO, 2007) - the fall of immunity 5-10 years after the last vaccine dose leaves susceptible adolescents and young adults, mainly due to decreased protective antibody titer (BRICKS, 2007). This reduction of immunity among adults may be related to the reduced circulation of *B. pertussis* in vaccinated populations as well, which causes less frequent exposure to antigens, and thus, less strengthening of natural immunity. (CAGNEY et al., 2008). This fact can be considered serious because some studies showed an increased risk of complications from the disease with age, including hospitalizations for pneumonia and sinusitis. (ISKEDJIAN et al., 2004). Moreover, adolescents and adults may serve as reservoirs for pertussis and often are the source of infection for children, beginning the cycle of transmission to newborns and infants not yet immunized, and the disease remains endemic. (LEE et al., 2004).

2.1.2 Pertussis toxin (PT)

Pertussis toxin (PT) is one of the most complex soluble bacterial proteins. (LOCHT, 2011). It is a multimeric 105 kDa protein (LOCHT, 2011) composed of five different

subunits, S1, S2, S3, S4 and S5. S1 subunit catalyzes the ADP-ribosylation of G proteins in target cells of mammals, while the subunits S2 to S5 (called oligomer B) are responsible for the binding of S1 to these cells. The ADP-ribosyltransferase enzymatic activity is associated with the domain located in the 180 N-terminal amino acids of S1 (termed C180), while the rest of this subunit is involved in G protein binding and interaction with the oligomer B. (KAMACHI; ARAKAWA, 2004)

Besides the enzymatic activity, mainly due to Arg9, Arg13 and Glu129 residues (PIZZA et al., 1989), subunit S1 is also recognized as the immunogenic portion of pertussis toxin once it promotes maturation of dendritic cells. These cells are responsible for activation of antigen-specific T and B cells which mediate adaptive immunity against *B. pertussis*. (HOU, 2003).

Studies have shown that this subunit expressed in *Mycobacterium bovis* BCG was able to induce protective immunity against the toxin itself and also against *B. pertussis* in a model of intracerebral challenge in mice. (NASCIMENTO et al., 2000). In addition, a DNA vaccine comprising a plasmid encoding this subunit was also able to induce immunity. (KAMACHI et al., 2003)

2.2 Vaccination

Due to the emergence of new production technologies and regulatory requirements, vaccine production has suffered constant and deep transformations. These new products, however, have high value and are causing technological dependence by developing countries such as Brazil (HOMMA et al., 2003), which leads to the requirement for short-term replacement of some vaccines, including DTP, used in the Brazilian public health system.

2.2.1 Celular vaccine

DTP vaccine is composed of tetanus toxoid (TT), diphtheria toxoid (DT) and a detoxified bacterial suspension of *Bordetella pertussis* (PRESTES, 2008). This vaccine has been shown to be effective with regard to immunization against pertussis, however, several adverse effects are related to this vaccine component. Among these effects can be included,local effects such as redness and swelling and systemic effects such as fever, vomiting, diarrhea and even seizures (SATO et al., 1984). Furthermore, it is known that these

effects are exacerbated with age and therefore the last dose of vaccine is given to five or six years of age.

2.2.2 Acelular vaccine

Therefore, in recent decades many groups started to study the possibility of producing a DTaP vaccine, containing only purified antigens of *B. pertussis*. A literature review conducted by Keitel (1999) showed that these acellular vaccines have been well tolerated and highly immunogenic in adolescents and adults. These vaccines with an antigenic content more than 50% lower than the formulations for children, and reduced adverse effects, demonstrated an estimated efficacy of 92% in a study among American teenagers (LEE et al., 2004).

Initially, the approach for production of this vaccine was bacterial culture and subsequent purification of some proteins recognized as being immunogenic. The major disadvantage of this procedure is that purification of components cause a low yield, since only that fraction of the cell is exploited for vaccine production. Studies have shown that only 1 dose of acellular vaccine can be produced from the same fermentation broth volume that would produce 10-20 doses of whole cell vaccine (HIGASHI et al., 2009). In addition, the vaccine generally has low immunogenicity due to chemical detoxification process by which must pass (PIZZA et al., 1989).

Today, there are several vaccines on the market that allow vaccination of adult and young babies' population (Annex I). However, these vaccines are produced by multinationals and too expensive to be acquired by the national health system of Brazil.

2.2.3 New strategies

Thus, current proposals for the production of DTaP consist in the use of recombinant technology that can be accomplished in two main ways. The first, already analyzed in the review of Loosmore and coworkers (1991), consists on the mutation of *Bordetella pertussis* to change certain amino acids in the S1 subunit of the toxin, responsible for toxicity thereof. The second, most studied currently, is the overexpression of components in microbial cells.

Burnette and coworkers (1988) have performed the cloning of all subunits of pertussis toxin in *Escherichia coli* in order to identify key functional sites and map epitopes to obtain candidates for an acellular vaccine. Furthermore, cloning attempts aimed not only at the production of pertussis toxin, but also other possible components of the DTaP vaccine have

already been performed in microorganisms such as *Bacillus subtilis* (OLANDER et al., 1991), *Pichia pastoris*, *Saccharomyces cerevisiae* (ROMANOS et al., 1991), *Bacillus brevis* (KOZUKA et al., 1996), besides insect cells (XU et al., 2011).

The advantage of using these organisms is especially the low cost of the process, mainly due to the high yield of protein (KOZUKA et al., 1996), and greater ease of purification. Furthermore, the recombinant production presents the advantage of eliminating the step of detoxification of the protein. (RAPPUOLI et al., 1992).

2.3 Cloning strategy

2.3.1 Escherichia coli

Escherichia coli, being one of the most known and utilized organisms for heterologous protein expression, is always the first option when intending to begin a cloning procedure. Specially because this bacterium offers a rapid and economic medium for production of recombinant proteins, and these advantages, combined with biochemical and genetical knowledge of this microorganism, have allowed its utilization for production of many molecules as insulin and bovine growth hormone.

However, some difficulties are found in the utilization of *E. coli* for immunogenic proteins production, mainly related to the correct folding of the protein that is directly connected to its antigenic properties. Another disadvantage of the therapeutical use of recombinant proteins produced in *E. coli* is the accumulation of lipopolysaccharide (LPS), generally designated endotoxin, which is pirogenic in humans and other mammals. Therefore, proteins need to go through a special purification process for become free of these endotoxins. (TERPE, 2006) Nevertheless, due to the many advantages that this system offers, it is indicated to test it in the first step.

2.3.2 pAE vector

The vector used in this work is the noncommercial vector pAE, kindly provided by Professor Odir Dellagostin (UFPel). This vector has several advantageous features, as: replication origin in *E. coli*, multiple cloning site, ampicillin resistance, strong promoter of phage T7, possibility of IPTG induction and inclusion of polyhistidine tail to the cloned protein. (Annex IV)

3 OBJECTIVES

3.1 General objective

Develop a reagent with the potential for use in acellular DTP vaccine (DTaP) made from a recombinant antigen produced by cloning and expression of an immunogenic protein of *Bordetella pertussis*.

3.2 Specific aims

- Cultive a strain of *Bordetella pertussis* to obtain biomass for DNA extraction;
- Cloning a portion of S1 subunit of pertussis toxin by PCR from the extracted DNA eliminating the enzymatic activity and, thus, the potencial pathogenicity of the molecule;
- Integrate the gene fragment in a plasmid that can be inserted and expressed in *Escherichia coli*;
- Execute the induction of protein expression and purification;
- Confirm the identity of the protein.

4 MATERIAL AND METHODS

4.1 Reactivation of strain, cultivation and inactivation of B. pertussis

The freeze-dried content of an ampoule of *B. pertussis* strain 137 (BP/T/01/99) obtained from TECPAR was homogenized with sterile saline solution (NaCl 0.9%), inoculated in an Erlenmeyer flask containing 50 mL of Stainer-Scholte medium (STAINER; SCHOLTE, 1971) (Annex II), and incubated on a shaker at 37°C and 150 rpm for 48 hours. Gram staining was performed to confirm identity and purity of culture. Furthermore, the strain was transferred to plates containing solid medium Charcoal Agar Base with Niacin (Annex II) and incubated at 37°C for 5 days, then, stored at 4°C.

Liquid cultures were distributed in 50 mL centrifuge tubes, inactivated in water bath at 60°C for 30 minutes and then centrifuged at 5000 xg for 20 minutes at 4°C for cell recovery. Pellets were stored at 4°C. (GUPTA et al., 1988)

All these procedures were performed in a biosecurity area in TECPAR.

4.2 Extraction and quantification of the DNA of B. pertussis

Pellet of one of the centrifuge tubes was used for DNA extraction that was developed combining freeze-thaw and phenol/cloroform/isoamil alcohol techniques (Annex III). It was read sample absorbance at 260 nm and an aliquot was applied to a 0.8% agarose gel and run at 80 V for 1 hour. Then, it was colored with Ethidium Bromide to verify the quality of DNA.

4.3 Primers design

The analysis needed for primers design intending properly amplification of the desired region of DNA and construction of the resulting plasmid *in silico* (Annex IV) was performed using the software Vector NTI (Invitrogen®).

ptxA gene sequence, encoding the S1 subunit of pertussis toxin was obtained from GenBank (GeneID: 2665068, locus tag BP3783) (Annex V). Aiming the directional cloning of amplicons into the expression plasmid, restriction sites for the enzymes *Bam*HI and *Hind*III were introduced in forward and reverse primers, respectively. The region selected for amplification contains 684 bp and excludes the first 44 aminoacids from the protein,

eliminating the potential pathogenicity of the molecule. Including the nucleotides inserted by the primers, the fragment size expected after PCR reaction is 698 bp.

4.4 Amplification of the gene of interest by PCR

PCR reaction was prepared following the GoTaq® Flexi DNA Polymerase (Promega) manufacturer's protocol, using 1 µL of 10 ng/µL DNA template for each 50 µL of reaction mix. The conditions of PCR were: 95ºC for 5 min (initial denaturation), 30 cycles of: 95ºC for 30s (denaturation), 55 ºC for 30s (annealing), 72ºC for 1 min (extension); 72ºC for 5 min (final extension). Efficacy of amplification was determined by running samples on an agarose gel 1.0% at 80 V for 1 hour.

4.5 Preparation of vector and gene for ligation

Both the gene obtained by PCR (after purification with GFX^{TM} PCR DNA and Gel Band Purification kit (GE Healthcare)) and the vector – pAE – were subsequently digested with restriction enzymes *Bam*HI (ThermoScientific) and *Hind*III (Promega) according to the manufacturer's protocol (Annex VI). Their concentrations were determined by spectrophotometry at 260 nm and the efficacy of digestion was determined in agarose gel 1.0%. The ligation reaction was executed with T4 DNA ligase (Promega) overnight at 4 ºC following the manufacturer's protocol. (Annex VII)

4.6 Transformation of E. coli DH5α for plasmid replication

The plasmids obtained were used to transform *E. coli* DH5α by heat shock (Annex VIII) for replication and verification of the success of ligation. In addition, a negative control was performed with pAE. The transformations were spread on Petri dishes containing LB medium supplemented with 100 mg/L of ampicillin (once the vector has the gene of resistance to this antibiotic) and incubated overnight at 37°C. The colonies observed on the plate were subjected to screening with phenol/chloroform to confirm the presence of the plasmid. 3 colonies were selected, which were inoculated in 5 mL LB medium with ampicillin, incubated overnight on rotatory shaker at 180 rpm and 37°C and used for the extraction of plasmid with illustra plasmidPrep Mini Spin kit (GE Healthcare).

To confirm the presence of inserts in the plasmids extracted, was performed a double digestion with the same enzymes and verified on an agarose gel 1.0%

4.7 Transformation of E. coli BL21 pLysS and induction of recombinant protein expression

The selected plasmid was utilized for transforming *E. coli* Bl21 pLysS expression cells. Cells transformed by heat shock (Annex VIII) were inoculated in LB medium containing ampicillin and 25 mg/L of chloramphenicol (to maintain the plasmid and pLysS) and incubated on rotatory shaker at 180 rpm overnight at 37°C. Cells without transformation also incubated in non-selective LB broth were utilized as negative control.

1 mL of this pre-inoculum was inoculated in 10 mL of LB medium with the same antibiotics and incubated on a rotatory shaker at 37°C and 180 rpm until the culture reaches optical density (OD) of 0.6 at 600nm. Then, induction of expression was performed with IPTG 0.3 mM for 3 hours. The confirmation of the recombinant protein expression was performed by electrophoresis SDS-PAGE on a 10% gel. (Annex IX).

4.8 Pre-inoculum production

The production of pre-inoculum has been performed in order to prevent new transformation would be required at each start of fermentation. So, transformed cells were stored at -80°C being stable for long periods.

For this purpose, 25 mL of transformed culture with OD of 0.5 was centrifuged at 3000 xg for 10 minutes at 4°C to recover the cells. The pellet obtained was resuspended in 2.5 mL of LB broth with antibiotics and 2.5 mL of sterile glycerol. The suspension was homogenized by vortexing and kept on ice while 200 µL aliquots were distributed into microcentrifuge tubes and stored at -80°C.

4.9 Solubility test to verify the presence of the protein in the supernatant or in inclusion bodies

1 mL of the pre-inoculum (see item 4.7) was used to inoculate 9 mL of LB broth with antibiotics and incubated overnight at 37°C and 150 rpm. Then, the culture was used to inoculate 100 mL of the same medium which was kept in shaker under the same conditions

until reach OD of approximately 0.8. IPTG was added to a final concentration of 0.3 mM and induction proceeded for 3 hours. The final culture was centrifuged at 7000 xg and 4°C for 15 min, the pellet obtained was washed with PBS (Annex X), centrifuged again under the same conditions and resuspended in 10 mL resuspension buffer. 4 pulses of sonication of 20s with the same time interval were performed. Then, another centrifugation at 15000 xg and 4°C for 10 min was performed to precipitate inclusion bodies. The supernatant was stored at 4°C and called soluble fraction. The pellet was resuspended in 10 mL solubilization buffer and was carried out a new cycle of sonication and centrifugation. The supernatant obtained was called insoluble fraction. Both fractions were applied to 10% polyacrylamide gel and submitted to Western Blotting analysis with anti-his6 antibody (Annex XI).

4.10 Cell growth and protein production kinetics

During fermentation process (see item 4.9) every hour were collected aliquots of 2 mL for prior verification of the influence of the induction time in cell growth and protein production. These fractions were centrifuged for 5 min at 11000 xg. The pellet obtained was resuspended in 200 µL of solubilization buffer (see item 4.9), passed through a cycle of four sonication pulses of 5s with the same time interval and again centrifuged under the same conditions. The supernatant was stored for further analysis in SDS-PAGE. To determine de growth rate (µ) at the different phases of the culture, it was used the following equation:

$\mu = (\ln x_t - \ln x_0) / (t - t_0)$; where, x_t and x_0 are the cell concentrations at times t and t_0, respectively. Cell concentrations were determined multiplying OD value by 0.35, once Lima (2004) described that for recombinant *E. coli* cultures, 1 U of absorbance at 600 nm corresponds to a cell concentration of 0.35 g/L, on a dry basis.

4.11 Optimization of expression

The most important factors when dealing with expression of recombinant proteins are the concentration of inducer (IPTG), the time and temperature of induction. To study these factors was carried out an experimental design of the type fractional factorial $3^{(3-1)}$ with three replicates at the center point. This type of design allows determining the statistical significance of each factor studied for the process. The levels studied of induction time were determined using the previous study described in section 4.10. The levels of other variables

were set as commonly used for recombinant protein expression in *E. coli*. (PAPANEOPHYTOU; KONTOPIDIS, 2012) (Table 1)

Table 1 - Factors and levels studied on expression optimization.

Factors/Levels	-1	0	1
Temperature (°C)	25	31	37
IPTG (mM)	0.1	0.5	0.9
Induction time (h)	2	3	4

One pre-inoculum of the cell bank (item 4.8) was used to inoculate 10 mL of LB medium with antibiotics for each experiment, and after an overnight growth, this culture was inoculated into 100 mL new LB broth with antibiotics. Fermentation and sampling was performed as described in 4.9. Samples were run on polyacrylamide gel at 10%. Gel image analysis was performed using the Image J software (NIH). The results were analyzed using the software Statistica 5.0.

4.12 Large-scale induction

Production of the recombinant protein for purification was performed in a final volume of 1 L following the same culture conditions as described on item 4.9. The cycles of sonication/centrifugation were performed until it was not observed any bacterial mass in suspension. This sample was named clarified fraction.

4.13 Protein purification

Purification was performed by affinity chromatography using a pre-packed column HisTrap FF 5 mL from GE Healthcare. These columns are filled with nickel resin which has affinity for polyhistidine tails present in the recombinant protein. 25 mL of the clarified fraction were injected in the column after balance with binding buffer. Elution was performed using imidazole, compound that has greater affinity for nickel then polyhistidine tails, thus being able to disconnect histidine from the resin. All buffers contained urea to ensure that inclusion bodies would be solubilized. Purification was performed on automated system ÄKTApurifier GE Healthcare. The protocols were adapted from those recommended by the manufacturer (GE Healthcare). (Annex XII).

After each chromatographic step, 150 µL of each fraction were aliquoted and stored at -80°C for subsequent dosage of total protein content (BRADFORD, 1976) and protein profile electrophoresis analysis.

4.14 Protein identification

4.14.1 DNA sequencing

Both forward and reverse strains of the insert were sequenced in the ABI-PRISM 3100 Genetic Analyzer (Applied Biosystems). Results obtained were compared with sequences of BLAST (NCBI) database to confirm that the correct fragment has been amplified.

4.14.2 Mass spectrometry

A protein band obtained by the SDS-PAGE was digested with trypsin (Promega) at 37°C for 24 hours. The sample was hydrolyzed in a mixed solution HCCA matrix-α-cyano-4-hydroxycinnamic acid Ⅱ. The mass spectrum was obtained using the spectrophotometer MALDI-TOF/TOF Autoflex II (Bruker Daltonics, Germany). Some signals present in the mass spectrum MS were fragmented in mode MS/MS. Peaks were analysed with the computer program FlexAnalysis 3.0 (Bruker Daltonics).

5 RESULTS AND DISCUSSION

5.1 Extraction of the DNA of Bordetella pertussis and amplification of the gene of interest by PCR

The average concentration of the DNA samples determined by spectrophotometry at 260 nm was approximately 3000 µg/mL (Annex XIII).

It was confirmed the fragment of the desired size (698 bp) and only one band in the sample, which evidences no nonspecific amplification (Figure 1). Furthermore, in negative control (NC) was not observed any band.

5.2 Preparation of vector and gene for ligation

Efficacy of digestion was proved, once at the pAE sample can be observed various bands and on pAE digested it can be observed only one band with the expected size (2822 bp). (Figure 2) Normally, on a noncut plasmid sample, it can be seen four different bands, corresponding to variations in DNA folding, once not only the size but also the shape affects migration in electrophoresis. When plasmid is cut, it migrates as linear DNA, so, the presence of only one band is expected. (SAMBROOK; RUSSEL, 2001)

On the other samples it can't be seen any difference, once the digestion eliminates only a few nucleotides and then presents no significant difference on molecule size.

5.3 Transformation of E. coli DH5α for plasmid replication

It was observed the growth of six colonies on the plate which was seeded with a culture transformed with plasmid pAE-S1. After screening by electrophoresis (figure 3), it can be observed that four colonies (P1, P2, P3 and P4) present the same pattern of running as negative control samples. P5 presents plasmid without insert, when compared with pAE sample. P6 presents a different pattern, although the plasmid seems to be smaller than pAE. Even so, this difference in running can occur due to variation in plasmid folding as described in item 5.3. So, P6 was previously chosen for plasmid extraction. Nevertheless, plasmids from P4 and P5 were also extracted for comparison after double digestion and thus, confirm the presence of insert.

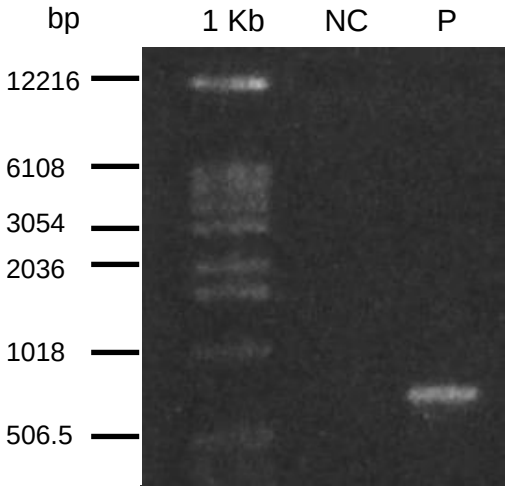

Figure 1 – Agarose gel 0.8% of polymerase chain reaction products.
1 Kb = molecular weight marker; NC = negative control of PCR; P = amplificated sample

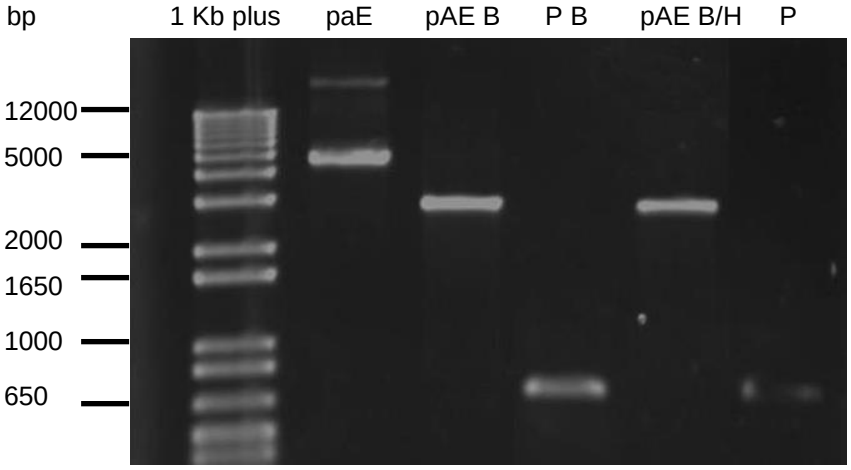

Figure 2 – Eletrophoresis in agarose gel 1.0% of the polymerase chain reaction products digested.
1 Kb plus = molecular weight marker; pAE = noncut vector; pAE B = vector after digestion with *Bam*HI; P B = amplicon digested with *Bam*HI; pAE B/H = vector after digestion with *Bam*HI *and Hind*III; P B/H = amplicon digested with *Bam*HI and *Hind*III.

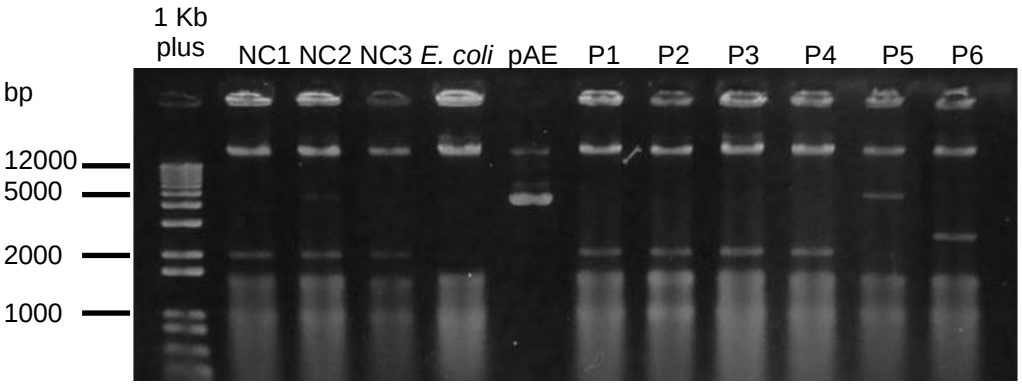

Figure 3 – Screening of colonies after transformation.
1 Kb plus = molecular weight marker; NC 1, NC 2, NC 3 = DNA extracted from 3 colonies of negative control; *E. coli* = DNA extracted from one *E. coli* DH5α colony; pAE = noncut vector; P1 until P6 = DNA extracted from the 6 colonies that had grown in plates with selective LB medium.

5.4 Confirmation of the presence of the insert in the plasmid obtained

Double digestion of the extracted plasmids confirmed the expected results (Figure 4), once only digestion of plasmid of the P6 colony has shown a band of 698 bp. Thus, this plasmid was chosen to transform *E. coli* Bl21 pLysS cells and produce the recombinant protein.

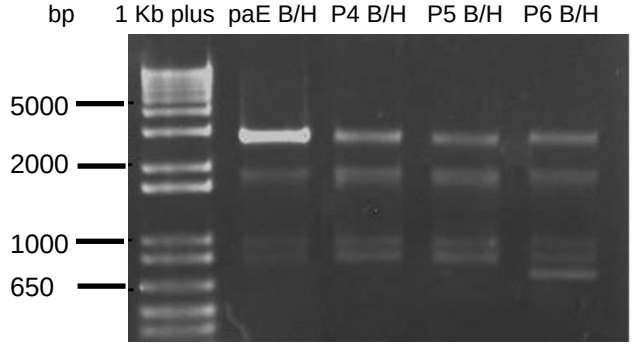

Figure 4– Double digestion of plasmid extracted from screening selected colonies.
1 Kb plus = molecular weight marker; pAE B/H = vector after digestion with *Bam*HI *and Hind*III; P4 B/H = plasmid digested with *Bam*HI and *Hind*III; P5 B/H = plasmid digested with *Bam*HI and *Hind*III; P6 B/H = plasmid digested with *Bam*HI and *Hind*III.

5.5 Confirmation of expression of the protein of interest

In SDS-PAGE (Figure 5) can be observed a band with approximately 28 kDa overexpressed in the inducted sample. This confirms the expression of the recombinant protein, and the efficiency of IPTG in the induction of protein expression. Although, it can be also seen a band with less intensity corresponding to the recombinant protein in non-inducted ample. This can show that the system of expression control, regulated by pLysS plasmid, is not completely efficient.

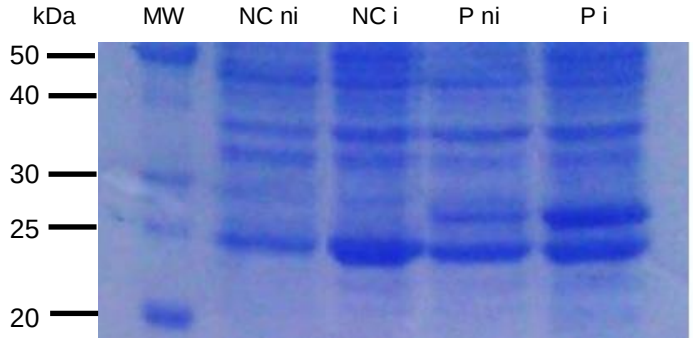

Figure 5 – Verification of recombinant protein expression.
MW = molecular weight marker; NC ni = negative control before induction; NC i = negative control after induction; P ni = transformed cells before induction; P i = transformed cells after induction.

5.6 Solubility test to verify the presence of the protein in the supernatant or in inclusion bodies

The solubility test has demonstrated the presence of the recombinant protein mainly in the insoluble fraction, in other words, in inclusion bodies (Figure 6) while a big part of other proteins are extracted in the soluble fraction. This is expected for recombinant proteins expressed in *E. coli* and this result was also obtained by Burnette (1988) that expressed recombinant S1 subunit of pertussis toxin at high phenotypical levels (7 to 22% of total cell protein content) in inclusion bodies. Choi and coworkers (2006) said that overexpressed proteins are often produced in the form of inclusion bodies and set several advantages in this fact, like: much higher levels of protein accumulation (usually greater than 25% of total proteins) than soluble proteins; inclusion bodies can be initially isolated in a highly purified and concentrated state by simple centrifugation leading to a significant decrease in downstream processing requirements for removing contaminating host proteins; resistance to proteolysis by *E. coli* proteases, allowing high-yield protein production. (CHOI, KEUM, LEE, 2006)

Nevertheless, this result can lead to some difficulties, once the protein needs to be refolded to be soluble in a common saline buffer and this process is not so easy to all proteins.

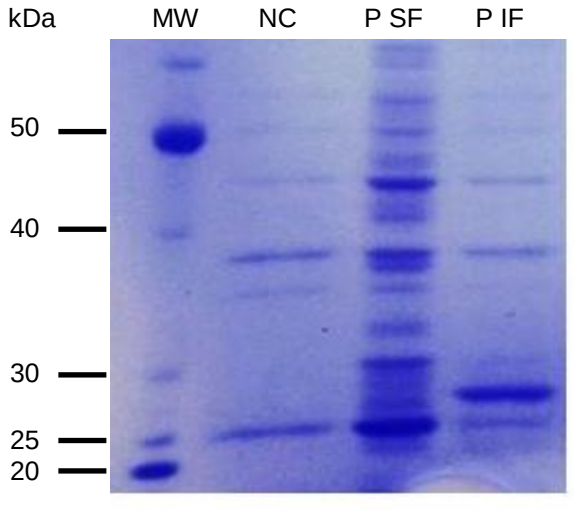

Figure 6 – Recombinant protein solubility.
MW = molecular weight marker; NC = negative control insoluble fraction; P SF = soluble fraction of recombinant cells; P IF = insoluble fraction of recombinant cells.

It was also confirmed the presence of the protein in the insoluble fraction by Western Blotting (Figure 7).

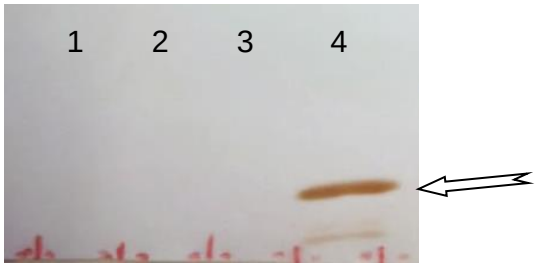

Figure 7 – Western blotting showing two fractions (soluble and insoluble).
1 = molecular weight marker (not recognized by anti-his6); 2 = negative control insoluble fraction; 3 = soluble fraction of recombinant cells; 4 = insoluble fraction of recombinant cells.

5.7 Cell growth and protein production kinetics

In the kinetic analysis of cell growth of cells of *E. coli* BL21 pLysS, lag phase was not observed for cells not transformed (NC). However, to cells transformed with the recombinant plasmid (P), lag phase was observed until the third hour of incubation. Only after 5 hours of cultivation, the absorbance at 600 nm reached an average value of 0.7, and was performed induction with IPTG. The exponential phase was observed from the third to half of the fifth hour of growth. (Figures 8 and 9) Following the addition of the inducing agent, was observed a decrease in growth rate, which was expected since overexpression overproduction of heterologous protein imposes metabolic burden stress, subsequently, cell growth decreases. (KIM et al., 2003)

The graphic of the natural logarithm of the cell concentration as a function of time represents the variation in growth rate (μ) (Figure 9). The calculated μ for each phase of the growth of transformed cells (P) is: 0.358 h^{-1} (lag phase), 0.626 h^{-1} (exponential phase) and 0.215 h^{-1} (after induction).

Cell concentration reached at the end of fermentation, both for the negative control, as for the transformed cells were approximately 0.42 g/L of fermentation broth.

Analysis of protein expression in each fermentation time indicates that the maximum expression was achieved at the end of fermentation, 3 hours after induction and that there was little change in other proteins present in the sample during this time (Figure 10). As the fermentation was not getting any samples after this time, it is unclear, in this experiment, whether a longer time would be even better for protein production.

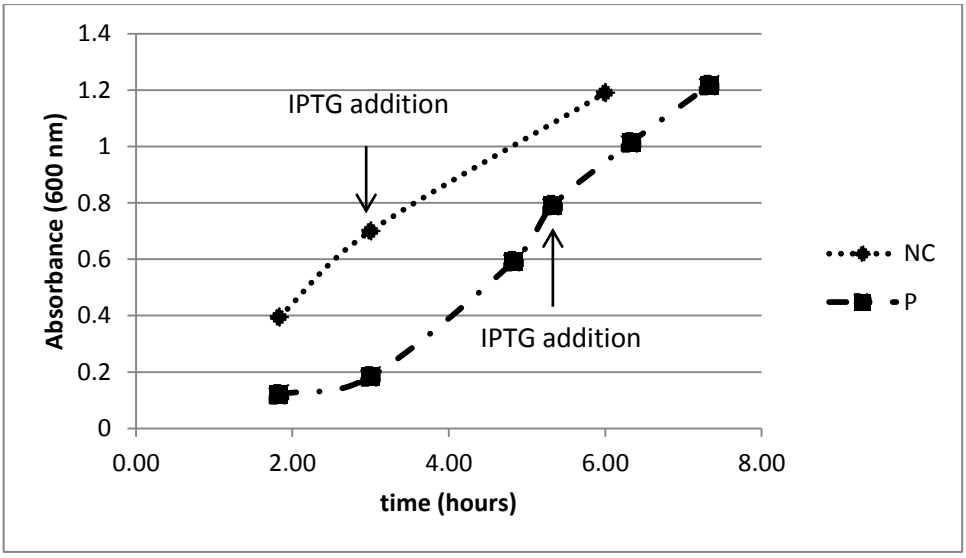

Figure 8 – Growth kinetics of transformed (P) and non-transformed (NC) cells before and after addition of induction agent (IPTG).

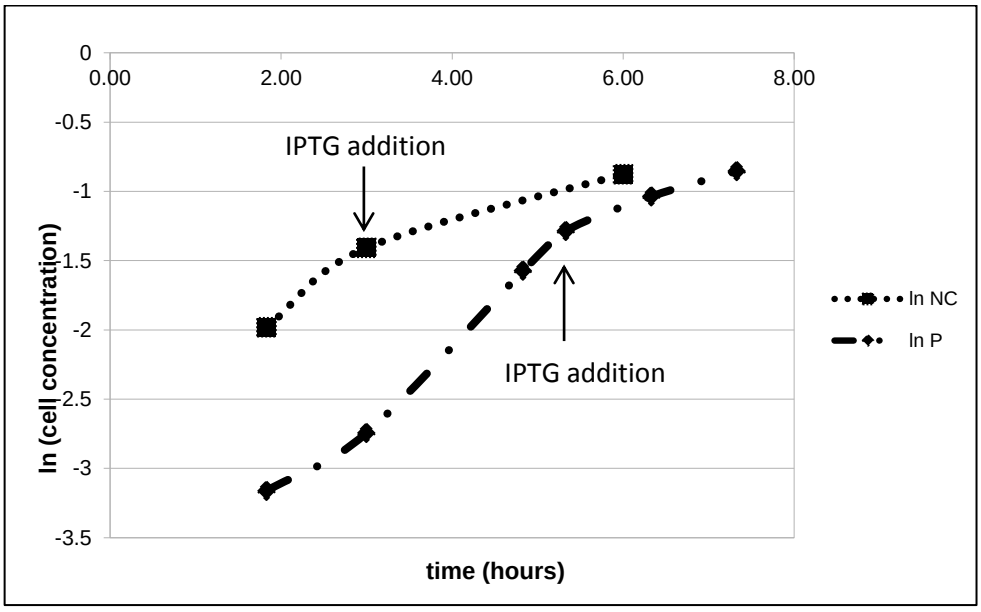

Figure 9 – Evaluation of growth phases before and after addition of induction agent (IPTG).

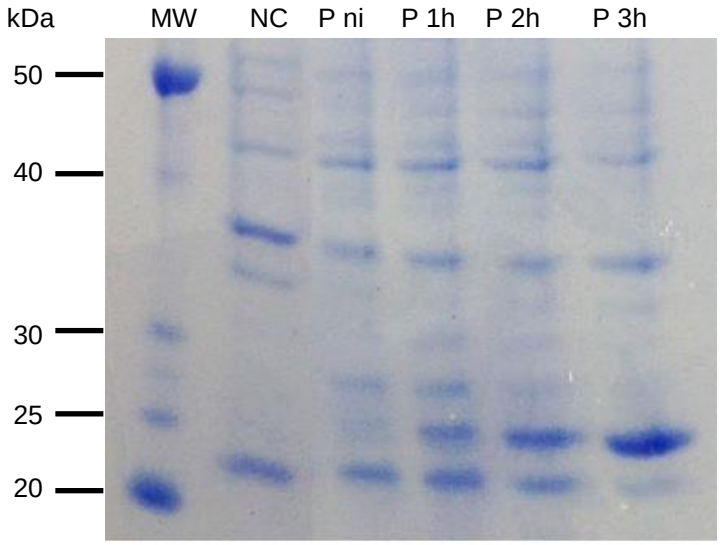

Figure 10 – Evaluation of influence of induction time in expression profile.
MW = molecular weight marker; NC = negative control; P ni = protein profile before induction; P 1h = protein profile after 1 hour of induction; P 2h = protein profile after 2 hours of induction; P 3h = protein profile after 3 hours of induction.

5.8 Optimization of expression

The analysis of the bands intensities in SDS-PAGE gel shows the best result was achieved in the experiment seven (in bold), where the conditions were: 37°C, 0,1 mM of IPTG and 3 hours of induction (Table 2). These results were obtained for the insoluble fraction of culture, once in the soluble fraction was not observed the recombinant protein band for any condition (data now shown).

Pareto diagram (Figure 11) of the experiment demonstrates that time of induction and IPTG concentration are statistic significant variables in expression of this protein, with p-value (significance level) of 0.05 or 5%. This diagram also shows that increase of IPTG concentration has a negative effect on protein production, i.e., this variable must be used in their lower value to achieve maximum expression values. This is very important when thinking about industrial processes, once IPTG is an expensive addictive, and if low concentrations give better responses, the costs are reduced.

The statistical significance of the model was checked and the equation coefficient (R^2) calculated was 0.935, suggesting that the fitted model could explain 93.5% of total variation.

The result obtained for the influence of IPTG concentration agree with that obtained by Cao and coworkers (2006) that have tested six different concentrations of this compound for induction of 1,3-propanediol oxidoreductase expression in *E. coli* and obtained the optimal value of 0.1 mM (the lower concentration tested).

Table 2 - Results of band intensities (peak area) obtained for the experimental design $3^{(3-1)}$ fractional factorial with 3 replicates of central point.

Experiment	**Factors**			
	Temperature	IPTG	Induction time	Peak area
1	-1	-1	-1	5202518
2	-1	0	1	256607
3	-1	1	0	5604640
4	0	-1	1	5232104
5	0	0	0	6442711
6	0	1	-1	5629640
7	**1**	**-1**	**0**	**8103196**
8	1	0	-1	5311104
9	1	1	1	268314
10 (C)	0	0	0	5967518
11 (C)	0	0	0	6971882
12 (C)	0	0	0	6135933

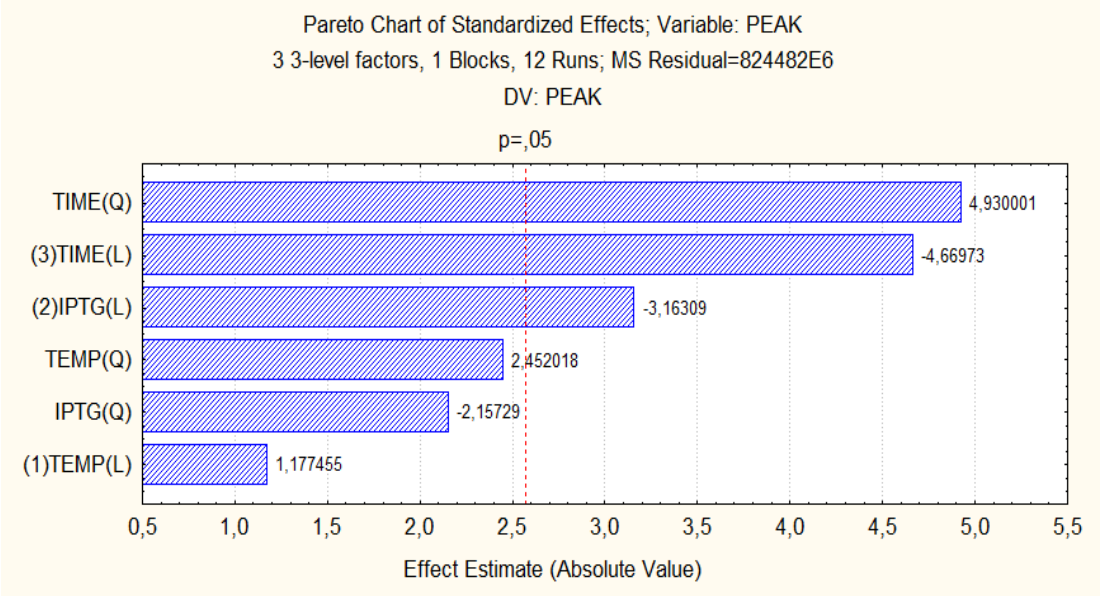

Figure 11 – Pareto diagram for induction conditions studied

5.9 Protein purification

SDS-PAGE of the samples acquired at purification of the clarified fraction (obtained as described in item 4.13) demonstrates that a prior purification could be done, but, even so, it has some proteins that were eluted together with the recombinant protein (Figure 13). This fact can be due to the low concentration of imidazole (5 mM) in the binding and washing buffer, once the column protocol recommends concentrations between 20 and 40 mM of this compound to decrease unspecific binding. Although, previous tests (data not shown) indicated that higher concentrations led to eluting the protein in binding and washing steps, fact that can also be seen in figure 12.

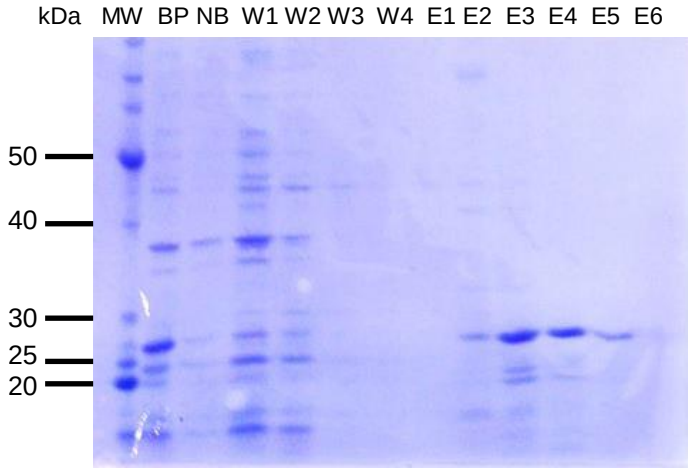

Figure 12 – Samples of purification process in HisTrap FF column.
MW = molecular weight marker; BP = sample before injection in column; NB = not-bounded proteins; W1, W2, W3 and W4 = fractions of column washing; E1, E2, E3, E4, E5, and E6 = eluted fractions.

Other strategies can be tested to improve purification, including decreasing NaCl concentration in all buffers, facilitating to the protein to bind the resin and then, increasing imidazole concentration to avoid unspecific binding.

Moreover, once the proteins with similar molecular weight has been yet excluded from the sample by the affinity chromatography, two steps of ultrafiltration with membranes of 20 and 35 kDa, or gel filtration chromatography could eliminate other proteins and also concentrate sample. It would also be useful to change buffer (to take off imidazole, urea and decrease salt concentration) and promote protein refolding, so it can be used for biological tests. Finally, the protein was eluted in 4 fractions (E2, E3, E4, and E5), and their concentrations, determined by Bradford analysis, in µg/mL, were, respectively: 133.73; 328.902; 272.176 and 91.364. So, the major fractions of the recombinant protein are the elutions three and four. Analysis of the gel image by software Image J (Annex XIV) gave the

proportion of the band of recombinant protein in function of total bands of each sample, and they are, respectively 54.28%, 71.53%, 75.21%, 95.79%. So, in these four fractions, it can be estimated that the total yield of protein obtained was 16.52 mg/L of total culture and, considering purity, this yield is estimated on 12 mg/L.

This result is more than 3 times higher than obtained by Buasri and coworkers (2012) that have constructed mutant *B. pertussis* strains to produce genetically inactivated pertussis toxin and achieved and expression of this protein of only 3.77 ± 0.53 mg/L of culture. Moreover, cultivation of *B. pertussis* requires a rich medium (see item 4.1) and, thus, is much more expensive than *E. coli* fermentation.

Another attempt to produce and fusion protein of subunits S1 and S3 of PT and FHA in *Streptococcus gordonii* has only achieved 12 µg of protein per liter of culture (LEE et al., 2002). Yet, the expression of S2 subunit of PT in *Bacillus brevis* has yielded 70 mg of protein per liter of culture (KOZUKA et al., 1996).

5.10 Protein identification

5.10.1 DNA sequencing

Comparison of the nucleotide sequence obtained from the cloned gene (Annex III) with BLAST database resulted in 99 matches with 99% of identity and E-value of 0.0. The lower the E-value, or the closer it is to zero, the more "significant" the match is. These matches were mainly of *Bordetella pertussis* pertussis toxin S1 subunit (ptxA) gene, as expected. There were results from *Bordetella parapertussis* and *Bordetella bronchiseptica* also. (Annex XV)

5.10.2 Mass spectrometry

Protein identification by mass spectrometry in MALDI-TOF has returned some hits according to pertussis toxin S1 subunit [*Bordetella pertussis*], but, not with so high scores (data not shown). This can be due to some contaminants in the sample after purification, which not led the equipment to distinguish between the peaks analyzed. Even so, once DNA fragment has also been sequenced and confirmed the correct protein, this result also confirms presence of this protein in the sample, although, it suggests that it needs better purification.

6 CONCLUSIONS AND PERSPECTIVES

By the proposed method, it was concluded that:

- It was possible the amplification of a specific DNA fragment (containing 684 bp) of the S1 subunit of pertussis toxin by PCR.

- This fragment, after digestion, insertion into specific vector and transformation into *E. coli* was able to be translated the S1 subunit of the pertussis toxin

- This expression was confirmed by SDS-PAGE once has been obtained a protein of approximately 28 kDa as expected, and *Western Blotting* with the anti-His6 antibody that recognized the same protein.

- The experimental design showed that the best conditions for inducing the expression of this protein have been the use of 0.1 mM IPTG lasting only 3 hours of induction at 37°C; although only IPTG and time were statistically significant factors.

- Production of a bank of transformed cells for use as pre-fermentation inoculum was efficient;

- The protein was expressed in insoluble form, the inclusion bodies were isolated and it has consisted in the first step of purification, then, this pre-purified suspension, were efficiently purified by affinity chromatography.

- The sequencing of DNA fragment and identification of the expressed protein have confirmed the satisfactory result of all cloning step

Thus, since it was succeeded in the expression of the antigen of interest, it is necessary to optimize purification process and check its biological activity through *in vitro* and *in vivo* to confirm its potential as acellular pertussis component of DTaP.

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8 ANNEXES

Annex I – Composition of main commercial DTaP vaccines

Component	KINRIX® (GSK)	PENTACEL® (Sanofi Pasteur)	TETRAXIM® (Sanofi Pasteur)	ADACEL® (Sanofi Pasteur)
Diphtheria toxoid (DT)	25 Lf	15 Lf	30 U.I.	2 Lf
Tetanus toxoid (TT)	10 Lf	5 Lf	20 U.I.	5 Lf
Pertussis toxoid (PT)	25 µg	10 µg	25 µg	2,5 µg
Filamentous hemagglutinin	25 µg	5 µg	25 µg	5 µg
Pertactin (69 kDa)	8 µg	3 µg	-	3 µg
Fimbriae	-	5 µg	-	5 µg
AGE INDICATION	4-6 years	6 weeks – 4 years	2 – 13 years	4 – 64 years
Source	Johns & Rutter (2010)	Johns & Rutter (2010)	Sanofi Pasteur Inc., 2004	Sanofi Pasteur Inc., 2011

Annex II – <u>Bordetella pertussis</u> culture medium composition

- Stainer-Scholte culture medium

Solution 1:

Component	Quantity
Tris base	6,06 g
Monosodium glutamate monohydrate	11,86 g
Proline	0,24 g
NaCl	2,5 g
KH_2PO_4	0,5 g
KCl	0,2 g
$MgSO_4$	0,126 g
$CaCl_2$	0,02 g
HCl 37%	3,4 mL
Deionized water	q. s. 1 liter

Solution 2:

Component	Quantity
L-cistine	0,4 g (dissolve in a few mililiters of HCl)
$FeSO_4.7H_2O$	0,1 g
Ascorbic acid	0,1 g
Niacin (nicotinic acid)	0,04 g
Glutathione reduced	1,0 g
Deionized water	q. s. 100 mL

The solution number 1 was sterilized by autoclaving. The solution number 2 was sterilized by filtration and added to solution 1 in the proportion of 10 mL/L.

- Charcoal Agar Base with Niacin medium:

Component	Quantity
Pancreatic digest of gelatina	2 g
Beef extract	2 g
NaCl	1 g
Starch	2 g
Nicotinic acid	0,0002 g
Charcoal	0,8 g
Agar	2,4 g
Deionized water	qsp 200 mL

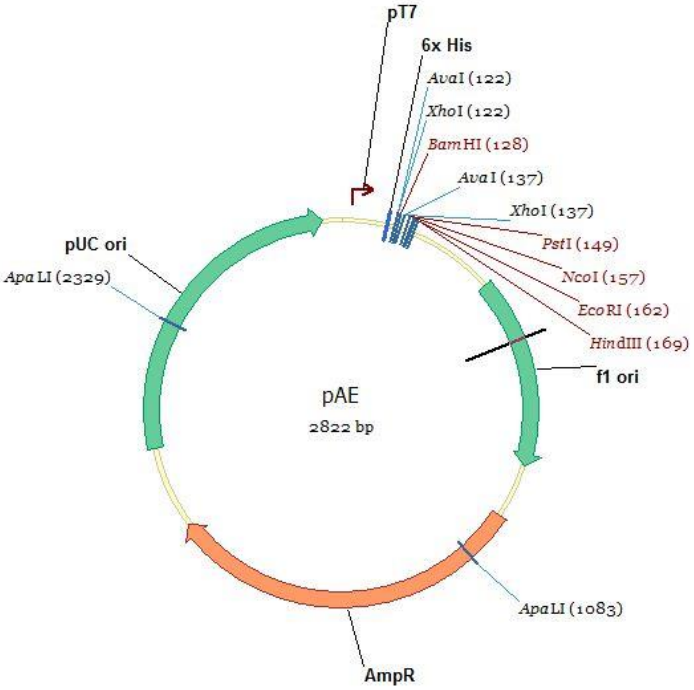
The medium was sterilized by autoclaving. After cooling, 10 mL of defibrinated blood was added.

Annex III - DNA Extraction protocol

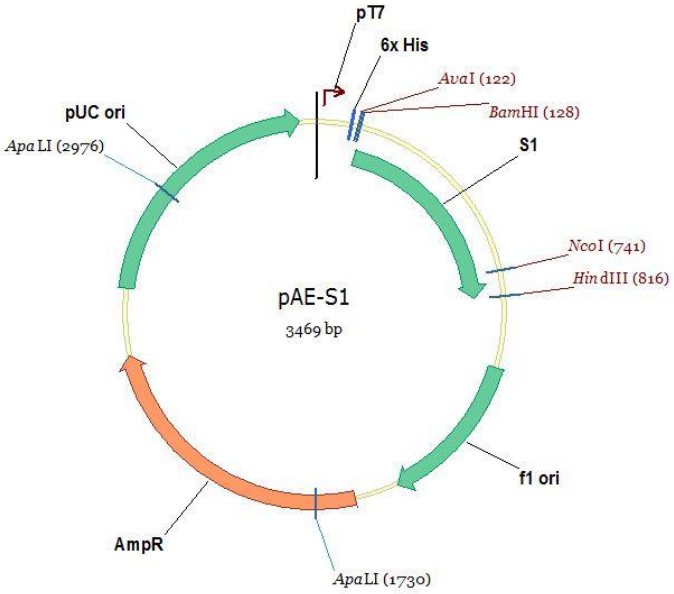
- Resuspend pellet in 500 µL in TE buffer by repeated pipetting (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0).
- Perform 3 freezing and thawing cycles (-70ºC, 5'; 80-95ºC, 2').
- Add 0.02 volume (at least 500 µL) of lysozyme (10 mg/mL) and incubate to 37ºC for 1 hour.
- Add 0.01 volume of SDS 10% and 0.01 volume of proteinase K (20 mg/ml). Mix thoroughly and incubate 1 h at 55°C.
- Warm up to 95ºC for 10' to denature proteinase K. Centrifuge 1000 xg, 5'.
- Add 0.01 volume of RNase (20 mg/mL) and incubate 37ºC, 30'.
- Add the same volume of phenol/chloroform/isoamyl alcohol (25:24:1) and gently mix thoroughly for 5' and spin 12000 xg, 5' in a microcentrifuge.
- Remove aqueous, viscous supernatant to a fresh microcentrifuge tube, leaving the interface behind. Add an equal volume of phenol/chloroform/isoamyl alcohol, extract thoroughly, and spin in a microcentrifuge for 5 min.
- Transfer the supernatant to a fresh tube. Add 0.1 volume sodium acetate 3M, pH 5.2 and 2 volume ethanol (-20ºC). Gently mix thoroughly.
- Spin 12000 xg, 30', 4ºC.
- Transfer the pellet to a fresh tube containing 300 µL of 70% etanol 9-20ºC). spin 12000 xg, 15', 4ºC . Repeat this step.
- Dry the pellet 37ºC.

Annex IV – pAE vector and resultant plasmid

- pAE vector



- Resultant plasmid



Annex V – Nucleotide sequence of the fragment cloned and primers

ptxA toxin subunit 1 [*Bordetella pertussis* Tohama I] - Gene ID: 2665068

1 atgcgttgca ctcgggcaat tcgccaaacc gcaagaacag gctggctgac gtggctggcg
61 attcttgccg tcacggcgcc cgtgacttcg ccggcatggg ccgacgatcc tcccgccacc
121 **gtataccgct atgactcccg cc**cgccggag gacgttttcc agaacggatt cacggcgtgg
181 ggaaacaacg acaatgtgct cgaccatctg accggacgtt cctgccaggt cggcagcagc
241 aacagcgctt tcgtctccac cagcagcagc cggcgctata ccgaggtcta tctcgaacat
301 cgcatgcagg aagcggtcga ggccgaacgc gccggcaggg gcaccggcca cttcatcggc
361 tacatctacg aagtccgcgc cgacaacaat ttctacggcg ccgccagctc gtactcgaat
421 acgtcgacac ttatggcgac aatgccggcc gtatcctcgc cgggcgcgct ggccacctac
481 cagagcgaat atctggcaca ccggcgcatt ccgcccgaaa acatccgcag ggtaacgcgg
541 gtctatcaca acggcatcac cggcgagacc acgaccacgg agtattccaa cgctcgctac
601 gtcagccagc agactcgcgc caatcccaac ccctacacat cgcgaaggtc cgtagcgtcg
661 atcgtcggca cattggtgcg catggcgccg gtgataggcg cttgcatggc gcggcaggcc
721 gaaagctccg aggccatggc agcctggtcc gaacgcgccg gcgaggcgat ggttctcgtg
781 tactacgaaa **gcatcgcgta ttcgttcta**g

Underlined nucleotides are the fragment cloned.

Bold nucleotides are the primer annealing region.

- Primers:

S1_for CAGGATCCCGCTATGACTCCCGCC

S1_rev GGAAGCTTAGAACGAATACGCGATGC

Underlined nucleotides are the restriction sites for *Bam*HI (S1_for) and *Hind*III (S1_rev)

- Fragment obtained by sequencing: > sequence exported from ACTGene560_E02.ab1

CGCCGGAGGACGTTTTCCAGAACGGATTCACGGCGTGGGGAAACAACGACAATG
TGCTCGAACATCTGACCGGACGTTCCTGCCAGGTCGGCAGCAGCAACAGCGCTTT
CGTCTCCACCAGCAGCAGCCGGCGCTATACCGAGGTCTATCTCGAACATCGCATG
CAGGAAGCGGTCGAGGCCGAACGCGCCGGCAGGGGCACCGGCCACTTCATCGGC
TACATCTACGAAGTCCGCGCCGACAACAATTTCTACGGCGCCGCCAGCTCGTACT
TCGAATACGTCGACACTTATGGCGACAATGCCGGCCGTATCCTCGCCGGCGCGCT
GGCCACCTACCAGAGCGAATATCTGGCACACCGGCGCATTCCGCCCGAAAACATC
CGCAGGGTAACGCGGGTCTATCACAACGGCATCACCGGCGAGACCACGACCACG
GAGTATTCCAACGCTCGCTACGTCAGCCAGCAGACTCGCGCCAATCCCAACCCCT
ACACATCGCGAAGGTCCGTAGCGTCGATCGTCGGCACATTGGTGCGCATGGCGCC
GGTGGTGGGCGCTTGCATGGCGCGGCAGGCCGAAAGCTCCGAGGCCATGGCAGC
CTGGTCCGAACGCGCCGGCGAGGCGATGGTTCTCGTGTACTACGAAAGCATCGCG
TATCGTTTTCTAAGCTTCC

Annex VI - Digestion reaction

*Bam*HI digestion:

Component	Vol
Ultrapure water	28 µL
5X Buffer	5 µL
DNA	15 µL
*Bam*HI	2 µL
TOTAL	50 µL

Overnight 37ºC

Purification with GFX™ PCR DNA and Gel Band Purification kit (GE Healthcare).

Recovered in 25 µL.

*Hind*III digestion:

Component	Vol
Ultrapure water	22.5 µL
5X Buffer	5 µL
BSA	0.5 µL
DNA	20 µL
*Hind*III	2 µL
TOTAL	50 µL

2 hours at 37ºC

Purification with GFX™ PCR DNA and Gel Band Purification kit (GE Healthcare).

Recovered in 25 µL.

Annex VII – Ligation reaction

	S1	pAE (negative control)
Vector	5 µL	5 µL
Insert	8 µL	--------
10X Buffer	2 µL	2 µL
T4 DNA ligase	1 µL	1 µL
Ultrapure water	4 µL	12 µL
TOTAL	20 µL	20 µL

Reaction mix was incubated overnight at 4ºC.

Annex VIII – Heat shock transformation protocol

Step 1: production of competent cells

- 10 isolated colonies of *E. coli* have to be grown in 20 mL LB medium at 37°C until A_{600}= 0.5 (about 3-4h)
- centrifuge the *E. coli* solution at 3000 xg at 4°C for 10 min in ice-cold sterile centrifugation tubes
- suspend the cell sediment in 20 mL ice-cold 0.1 M $MgCl_2$ solution
- centrifuge at 3000 xg at 4°C for 10 min
- suspend the cell sediment in 20 ml ice-cold 0.1M $CaCl_2$ solution and incubate for 30 min at 4°C
- centrifuge at 3000 xg at 4°C for 10 min
- suspend the cell sediment in 1 ml ice-cold 0.1 M $CaCl_2$ solution
- add 100 µL glycerol (sterilized by autoclaving) and freeze the cell suspension at -80°C in 100 µL

Step 2: transformation of the competent cells

- thaw the suspension with the competent *E; coli* cells on ice (about 15 min)
- add 20 µL competent *E. coli* cell solution to 5 ng (2 µL) plasmid DNA
- incubate for 30 min on ice
- incubate for 90 s at 42°C
- incubate for 2 min on ice
- add 200 µL LB medium
- incubate at 37°C for 2h while agitating
- transfer to 5 mL of selective LB medium (with the necessary antibiotics) or spread in Petri dishes contained the same medium
- incubate at 37°C overnight

Annex IX – SDS-PAGE

- 10% resolving gel

	1 gel (10 mL)	**2 gels (15 mL)**
Purified water	4.0 mL	5.9 mL
30% acrylamide mix	3.3 mL	5.0 mL
1.5 M Tris (pH 8.8)	2.5 mL	3.8 mL
10% SDS	0.1 mL	0.15 mL
10% ammonium persulfate	0.1 mL	0.15 mL
TEMED	0.004 mL	0.006 mL

- 5% Stacking gel

	1 gel (2 mL)	**2 gels (4 mL)**
Purified water	1.4 mL	2.7 mL
30% acrylamide mix	0.33 mL	0.67 mL
1.5 M Tris (pH 8.8)	0.25 mL	0.5 mL
10% SDS	0.02 mL	0.04 mL
10% ammonium persulfate	0.02 mL	0.04 mL
TEMED	0.002 mL	0.004 mL

Adapted from: SAMBROOK; RUSSEL, 2001

Annex X – Buffers composition

PBS:
137 mM Sodium chloride
2.7 mM Potassium chloride
10 mM Disodium hydrogen phosphate
1.8 mM Potassium dihydrogen phosphate
pH 7.4

Resuspension Buffer:
20 mM NaH_2PO_4
pH 8.0

Solubilization Buffer:
20 mM NaH_2PO_4
500 mM NaCl
6M Urea
pH 8.0

Annex XI - Western Blotting protocol

- After electrophoresis, note gel dimensions, mark the beginning and equilibrate the gel in transfer buffer (10-30 min)
- Preparing the membrane: wet in 100% methanol (15 s), and rinse with ultrapure water for 15-20 s; balance in transfer buffer for at least 5 min;
- Soak the filter paper and sponges in transfer buffer for at least 30 seconds;
- To assemble the sandwich, follow the order: negative support, sponge, filter paper, gel, membrane, filter, sponge, positive support.
- Remove bubbles between every layer of the sandwich
- Place the sandwich in the vessel, add transfer buffer and connect the power. Transfer at 24 V and 4 ° C overnight.
- Increase the voltage to 48 V and held for 1 hour.
- Remove the membrane and rinse it with distilled water;
- Block the membrane in blocking buffer for 1 hour with gentle agitation.
- Incubate the membrane with anti-His (GE Healthcare) primary antibody diluted 1:3000 in blocking buffer
- Incubate for 1 hour at room temperature with gentle agitation
- Wash the membrane three times for 5 minutes with wash buffer.
- Incubate the membrane with anti-mouse secondary antibody diluted 1:10,000 in wash buffer.
- Wash the membrane three times for 5 min with wash buffer and then wash 2 times with PBS 5 minutes.
- Incubate with revealer for 1-5 minutes to observe the reaction
- Stop with PBS.

Blocking buffer: 10% (w/v) non-fat dry milk, 1x PBS, 0.05% Tween-20

Wash buffer: 1x PBS, 0.05% Tween-20

Reaveler: 10 mg DAB (3,3′-Diaminobenzidine tetrahydrochloride) in 15 mL of buffer (50 mM Tris, 0.15 M NaCl, pH 7.6)

Transfer buffer: 25 mM Tris, 192 mM glycine, 20% methanol

Annex XII – Protein purification protocol

Sample	25 mL sample of the clarified fraction
Flow rate	5 mL / min
Binding buffer	20 mM NaH_2PO_4 500 mM NaCl 6 M urea 5 mM imidazole pH 7.4
Elution buffer	20 mM NaH_2PO_4 500 mM NaCl 6 M urea 500 mM Imidazole pH 8.0
Steps	1. Balance with 5 CV of binding buffer; 2. Sample application; 3. Washing with binding buffer until the absorbance at 280 nm reached zero; 4. Gradient between elution and binding buffer in 5 CV
Fractions	Not-bounded sample Washing samples: 15 mL/fraction Elution samples: 5 mL/fraction

Annex XIII – Determination of extracted DNA concentration

Results of absorbance at 260 nm and DNA concentration (µg/mL) calculated for 2 samples (P1 and P2) diluted 100 times of DNA extracted from *Bordetella pertussis.*

Sample	Abs_{260} (1:100)	[DNA] (µg/mL)
P1	0.732	3660 µg/mL
P2	0.543	2715 µg/mL
Average	-	3187 µg/mL

Concentration of DNA obtained was determined by the following equation:

$$[DNA]\ (\mu g/mL) = Abs_{260} \times 50 \times FD$$

Where: [DNA] is the DNA concentration in µg/mL; Abs_{260} is the absorbance measured at 260 nm, and FD is the dilution factor of the sample.

Annex XIV – Image J analysis

Image J software builds a graphic with intensity of all bands present in each fraction of the electrophoresis gel. From this graphic, can be selected the peaks and quantified the area of each one. The table show values for each band viewed in the gel image. In bold is represented the band of the recombinant protein, and the value of this band divided for the sum of all fractions gives the percentage of this protein in the sample.

Sample	Peak	Area	Purity
E2	1	128485	
	2	524335	
	3	182556	
	4	144192	
	5	**1719385**	54.28%
	6	468556	
	Total	3167509	
E3	1	89071	
	2	188607	
	3	**6225497**	71.53%
	4	1153042	
	5	751385	
	6	296314	
	Total	8703916	
E4	**1**	**5085548**	75.21%
	2	236728	
	3	1439861	
	Total	6762137	
E5	**1**	**3285355**	95.79%
	2	144485	
	Total	3429840	

Annex XV – Results obtained by comparison of the cloned gene sequence with NCBI database

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Bordetella pertussis strain 475 pertussis toxin subunit 1 precursor (ptxA) gene, complete cds	1219	1219	98%	0.0	99%	KC992321.1
Bordetella pertussis strain BP130 pertussis toxin S1 subunit (ptxS1) gene, partial cds	1219	1219	98%	0.0	99%	GQ250917.1
Bordetella pertussis strain BP112 pertussis toxin S1 subunit (ptxS1) gene, partial cds	1219	1219	98%	0.0	99%	GQ250910.1
Bordetella pertussis strain BP105 pertussis toxin S1 subunit (ptxS1) gene, partial cds	1219	1219	98%	0.0	99%	GQ250906.1
Bordetella pertussis ptxS1 gene for pertussis toxin S1 subunit, ptxS1D allele	1219	1219	98%	0.0	99%	AJ506996.1
Bordetella pertussis gene for pertussis toxin subunit S1 (s1D allele)	1219	1219	98%	0.0	99%	AJ245368.1
Bordetella pertussis gene for toxin subunit S1	1219	1219	98%	0.0	99%	X16347.1
Bordetella pertussis 18323 complete genome	1208	1208	98%	0.0	99%	HE965805.1
Bordetella pertussis strain BP115 pertussis toxin S1 subunit (ptxS1) gene, partial cds	1208	1208	98%	0.0	99%	GQ250912.1
Bordetella pertussis strain BP114 pertussis toxin S1 subunit (ptxS1) gene, partial cds	1208	1208	98%	0.0	99%	GQ250911.1
Bordetella pertussis strain BP102 pertussis toxin S1 subunit (ptxS1) gene, partial cds	1208	1208	98%	0.0	99%	GQ250903.1
Bordetella pertussis toxin gene encoding subunit S1, strain CZ	1208	1208	98%	0.0	99%	AJ006159.1
Bordetella pertussis toxin gene encoding subunit S1, strain 18323	1208	1208	98%	0.0	99%	AJ006151.1
Bordetella pertussis strain 267 pertussis toxin subunit 1 precursor (ptxA) gene, complete cds	1203	1203	98%	0.0	99%	KC992320.1
Bordetella pertussis strain 39 pertussis toxin subunit 1 precursor (ptxA) gene, complete cds	1203	1203	98%	0.0	99%	KC992319.1
Bordetella pertussis CS, complete genome	1203	1203	98%	0.0	99%	CP002695.1
Bordetella pertussis strain BP150 pertussis toxin S1 subunit (ptxS1) gene, partial cds	1203	1203	98%	0.0	99%	GQ261920.1
Bordetella pertussis strain BP139 pertussis toxin S1 subunit (ptxS1) gene, partial cds	1203	1203	98%	0.0	99%	GQ261909.1