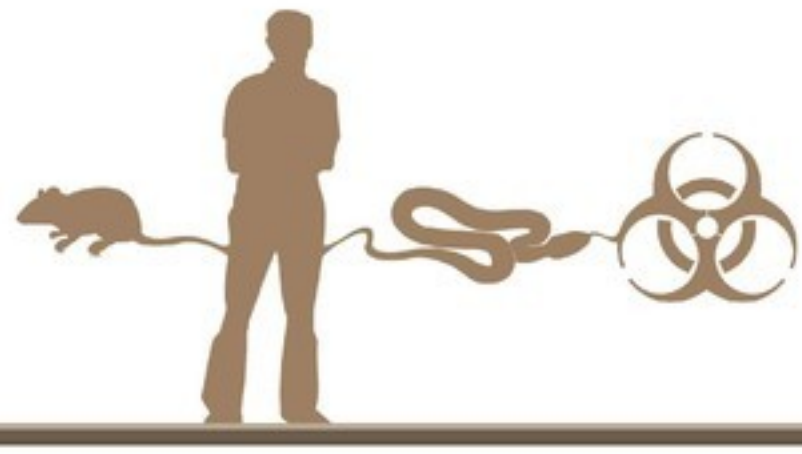
6. Biotechnology



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6.01 Characterization of the recombinant leptospiral protein LIC13417 expressed in *Escherichia coli*

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Introduction: Leptospirosis is an important zoonotic disease, widely spread around the world caused by spirochetes of the genus *Leptospira*. The sources of leptospires are essentially wild and domesticated mammals harboring the spirochetes in the urinary system. With the sequencing of the genome of *L. interrogans*, several genes encoding putative surface proteins were identified. These proteins are potential targets for inducing immune response during infection and may also mediate the initial adhesion process to host cells. **Objectives:** This study aims to biochemically characterize the protein encoded by the gene LIC13417 and to evaluate its immune response activity in animal model **Methods:** The secondary structure of the recombinant protein was analyzed by circular dichroism (CD) spectroscopy. Surface exposure of the native protein in *L. interrogans* was evaluated by proteinase K (PK) accessibility. Four-week-old BALB/c mice were immunized subcutaneously three times, at 2-week interval, bled from the retro-orbital plexus and the antibody response evaluated by ELISA and Western blotting. The animals were sacrificed and the splenocytes isolated for evaluation of lymphocyte proliferation and cytokine profiles in response to prime boosted antigen. The capacity of this protein to mediate attachment to ECM and serum components was evaluated by ELISA binding assays. **Results and Discussion:** The obtained CD spectrum for the recombinant protein suggests a predominance of beta-sheet. Our data suggest that the native protein is surface exposed, as demonstrated by PK degradation results. Animal immunization with the rLIC13417 protein elicited a Th2 response, as revealed by the increase in antibody titers during subsequent boosters. A moderate Th1 response was also stimulated, as shown by lymphoproliferation and production of IFN-gamma cytokine. The recombinant protein showed a dose-dependent attachment to plasminogen while no adhesion to extracellular matrix components was detected. Surface exposure and binding to plasminogen suggest the involvement of the protein LIC13417 in the infection process.

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6.02 Durvenoy's (venom) gland transcriptome of a false coral snake *Phalotris mertensi* (Serpentes: Colubridae) reveals an acid lipase as a putative new venom component

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Introduction: Colubrids comprises the majority of snake species. Despite of this fact, this is a group that receives less attention in the field of Toxinology because their bites are, with few exceptions, nonlethal to humans because of their inability to deeply inject the venom. However, few studies on the characterization these venoms revealed a repertoire of potentially important biomolecules. **Objectives:** In order to identify new toxins and to understand the pharmacological effects and the evolutionary aspects of such venoms, a transcriptome analysis was performed on *P. mertensi* venom glands and a putative new toxin cDNA was selected for recombinant production. **Methods:** Venom glands from specimens of *P. mertensi* were obtained and total RNA was extracted. Messenger RNA purification was performed using a column of oligo-dT cellulose and the cDNAs were synthesized from 5 μg of mRNA using the Superscript Plasmid System for cDNA Synthesis and Cloning. cDNAs were directionally cloned in the pSPORT-1 plasmid and transformed in *Escherichia coli* DH5α electrocompetent cells. Plasmid DNA was isolated using alkaline lysis from randomly chosen clones. DNA was sequenced on an ABI 3100 sequencer using the BigDye 3.1 kit with standard 5' primer (M13R). The clone corresponding to acid lipase was selected for expression in *E. coli*. **Results and Discussion:** A total of 1385 ESTs (expressed sequence tags) were generated and grouped into 777 clusters. About 37% of all transcripts code for putative venom components. Interestingly, the transcriptome analyses showed an abundant presence of the acid lipase (~7.5%) not reported in the composition from the venom gland of other snake families.The recombinant acid lipase was obtained as a histidine tag fusion protein in *E. coli* BL21 Star™ (DE3) pLysS, using pAE vector. The protein was purified by affinity chromatography using an immobilized metal resin and now is subject to the generation of antibodies seeking to verify if the protein is in fact secreted to the venom. Besides this unusual molecule, nine other toxin classes generally found in typical venomous snakes were also detected in *P. mertensi*. Among them, metalloproteinases represent the major protein type.

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6.03 Characterization and biological effects of a recombinant sphingomyelinase D from *Loxosceles gaucho*

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[1]Laboratório de Imunopatologia, [2]Laboratório de Fisiopatologia, Instituto Buntatan, Brazil

Introduction: The clinical picture caused by *Loxosceles* spider venom is characterized initially by an intense local inflammatory reaction and subsequent presence of dermonecrosis at the bite site. Phosfolipases D (PLDs) present in the venoms of *Loxosceles* spiders are the main components responsible for the local and systemic effects of whole venom. Many studies have characterized several biological effects of *Loxosceles gaucho* venom, but despite of its importance, to date no PLDs from this spider have been cloned and expressed to perform biological characterization of these molecules. Therefore, recently we have cloned and expressed a phospholipases D (LgRec1) from *L. gaucho*. **Objectives:** To characterize the biological activities of recombinant LgRec1 and to perform neutralization assays with antibodies raised against this toxin. **Methods:** Local reaction and dermonecrotic activity caused by LgRec1 was assayed by i.d. injection of 3.0 μg of *L. gaucho* venom (positive control) or LgRec1 into rabbit epithelium of the dorsal flank. Human platelet-rich plasma aggregation due to whole venom or recombinant toxins (10 mg/ml) were determinated using a Chrono-Log Whole Blood Aggregometer. Imuno cross reactivity of LgRec1 was performed by ELISA and Western blot. Neutralization of LgRec1 local reaction and dermonecrotic activities was performed incubating LgRec1 separately with Antiarachnidic serum (AAS), monoclonal antibody $MoALg_1$ produced against the dermonecrotic component of *L. gaucho* venom, anti-*L. gaucho* serum or anti-LgRecDT1 serum (0.2 ml) for 30 min at 37°C and injected i.d. into the rabbit dorsum. Neutralization of local reaction and dermonecrotic activities of *L. gaucho* venom by anti-RecDT1 serum was performed incubating 1.5, 3 and 6 μg of crude venom separately (30 min at 37°C) with 200 μL of anti-LgRecDT1serum and injected i.d. into the rabbit dorsum. **Results and Discussion:** ELISA and Western blot assay revealed evidences that LgRecDT1 belongs to the dermonecrotic toxin family, since it cross-reacted with the $MoALg_1$. Using human platelet-rich plasma, LgRec1 showed to promote platelet aggregation at concentration as low as 5 μg/mL. AAS, MoALg1 antibody, anti-*L. gaucho* venom and anti-LgRec1 were able to neutralize above 70% of local reaction and completely the dermonecrotic activity. Similarly, incubation of whole venom with anti-LgRec1 serum showed inhibition of local reaction (around 70%), while the dermonecrosis was almost completely abolished in all tested doses. Taken all together the results presented here may contribute to the knowlwdge of the mechanisms of venom action and also shed light to the understanding of the diversity of PLDs toxins from *Loxosceles gaucho* venom.

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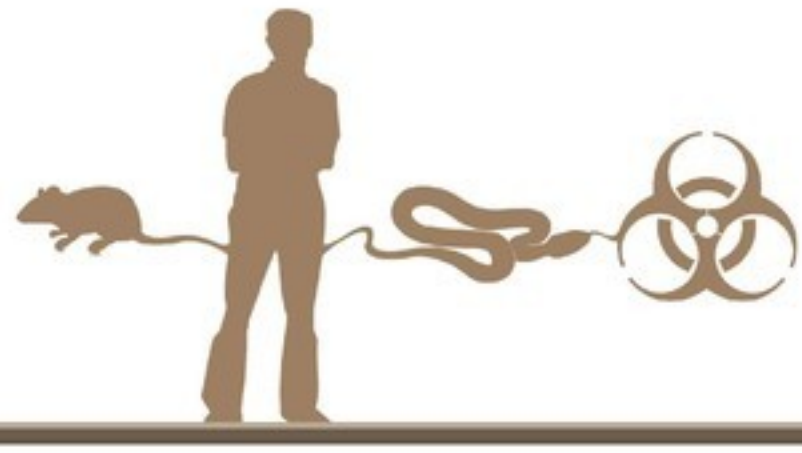

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6.04 Effects of temperature and pH on growth of *Haemophilus influenzae* type b and on the stability of its capsular polysaccharide

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Introduction: *Haemophilus influenzae* type b (Hib) is a Gram negative bacterium that causes severe infection in infants and elderly, such as pneumonia, septicemia and meningitis. Vaccine for Hib infections is produced using the capsular polysaccharide of the bacterium conjugated to a carrier protein. This polysaccharide (PRP) is a polymer of ribitol and ribose units, linked by a phosphodiester bond that shows similarity to the one in ribonucleic acid. This bond is likewise susceptible to alkaline hydrolysis, and the rate of hydrolysis increases as both pH and temperature increase. This event is responsible for the instability of the molecule and results in the low recovery ratios observed in the purification steps. **Objectives:** This work explores the dependence of bacterial growth, polysaccharide production and polysaccharide stability in different combinations of pH and temperature in order to identify the optimal cultivation conditions, with the balance between maximum polysaccharide productivity and minimum degradation. **Methods:** *H. influenzae* type b strain GB3291 was provided by Adolfo Lutz Institute. Cultivations were conducted on the modified MMP medium, on a 13L bioreactor, in the temperature range of 29 to 37°C, and in the pH range of 6.5 to 7.5. Polysaccharide content was assayed by Bial's modified method, and the molecular mass was measured by HPSEC. **Results and Discussion:** The optimal conditions in the range studied were defined at 32°C and pH 7.0. Polysaccharide productivity was determined as 3.92 mg/L/h per gram of glucose consumed, whereas the productivity in the standard conditions (37°C, pH 7.5) was only 3.6% higher; the molecular mass in the optimal conditions (980kDa) was twice as big as in the standard ones (490kDa). The higher molecular mass resulted in an improvement in the downstream process, increasing the recovery ratio of the first purification step from 50% to more than 80%. In the overall fermentation kinetics, the maximum specific growth rate was reduced with decreasing temperature, but increased in the more acidic pH; specific rate of polysaccharide formation decreased with temperature, while pH had little or no effect; molecular mass, which was assayed based on similar polysaccharide concentrations, increased significantly at lower temperatures and pH. These results support that, despite of the fact that the standard conditions represent the maximum polysaccharide productivity, lowering the fermentation parameters is an interesting strategy to obtain a more satisfactory global productivity, contributing for greater recovery ratios in the downstream process.

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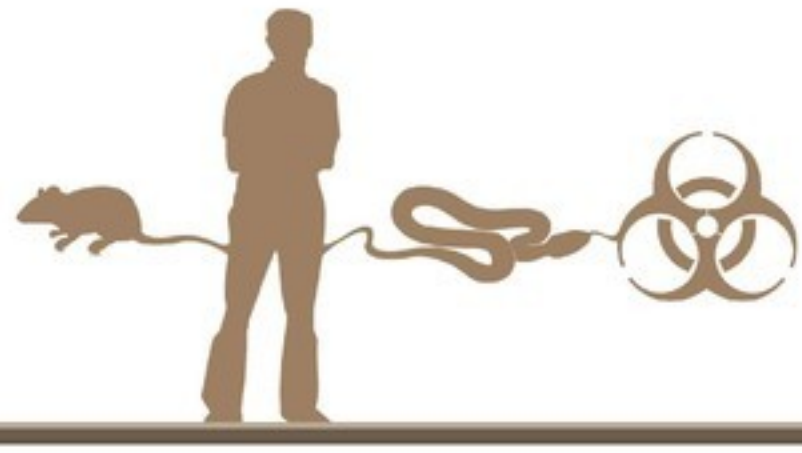

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6.05 Selection of family 2 PspA molecules with broad-ranging serocross-reactivity

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Introduction: *Streptococcus pneumoniae* is the pathogen responsible for the most cases of pneumonia, septicemia, bacterial meningitis and otitis media worldwide. The Pneumococcal Surface Protein A (PspA) is a promising vaccine candidate against this pathogen. It is a surface exposed protein that is capable of inducing the production of protective antibodies. Because it presents some structural diversity, at least one fragment of PspA from each major Family of PspA (1 and 2) should be included in an effective PspA-based vaccine and it has been shown in previews studies that PspAs from different clades present variable degrees of cross-reactivity. **Objectives:** This study has as its major objective to determine, from a panel of Brazilian pneumococcal isolates, which is able to induce the highest level of cross-reactivity within Family 2, which is composed by clades 3, 4 and 5. **Methods:** 15 Pneumococcal strains containing family 2 PspA (gently provided by Universidade Federal de Goiás or Instituto Adolfo Lutz) were used for PCR amplification of *pspA* fragments; sequencing was used to confirm the families and clades. The *pspA* sequences were then inserted into the pAE-6xhis expression vector. This vector was used because it provides a reasonable expression level for PspA. The final constructions (vector plus *pspA* sequence) were used for transformation into *E.coli* BL21 DE3. The resultant bacteria were induced with IPTG for expression of the recombinant PspAs; the soluble proteins were purified by nickel affinity chromatography providing 6 different rPspA molecules. BALB/c mice were immunized with these rPspA and sera from these animals were evaluated for their serocross-reactivity. **Results and Discussion:** The first analysis performed was a Western blot against a panel of diverse pneumococcal strains bearing PspAs from family 2: clades 3, 4 and 5. Then, the 4 most broad-ranging sera were selected. These 4 sera were used to evaluate their ability to bind to the pneumococcal surface as analyzed by FACS analysis. As a result, the best 2 sera were selected, which will be evaluated as to their ability to increase the deposition of complement on the pneumococcal surface, also by FACS analysis. Determination of opsonophagocytic activity with the same panel will be performed to confirm serocross-reactivity. These analyzes showed that even antibodies raised against the same rPspA clades induce different levels of cross-reactivity. The preliminary results showed that PspAs from clade 4 produce more cross-reactive sera. Finally, it will be possible to select a molecule capable of inducing a high level of cross-reactivity.

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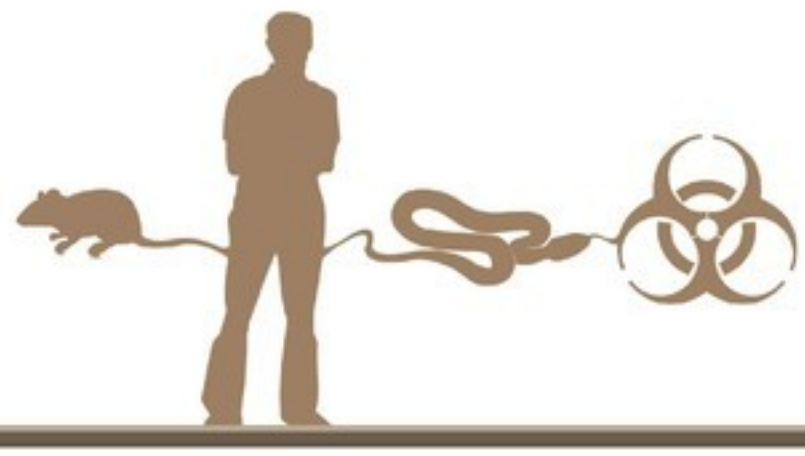

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6.06 Possible role of the protein Lsa33 of *Leptospira interrogans* in host-pathogen interaction

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Introduction: Leptospirosis is a zoonosis of global importance that is being considered an emerging infectious disease. Studies have been conducted to characterize novel antigens. **Objectives:** Our goal is to express and characterize the surface protein of *L. interrogans* serovar Copenhageni encoded by the gene LIC11834. **Methods:** Bioinformatics analysis of the gene sequence; design of primers; genomic DNA extraction and RNA extraction and amplification by PCR (study of conservation); cloning of PCR product in expression vector; expression and purification of recombinant protein; protein analysis by circular dichroism spectroscopy; production of polyclonal antibodies by mice immunization; evaluation of the capacity of this protein to mediate attachment to ECM and components of human serum by binding assays; analysis of the cellular localization of the protein. **Results and Discussion:** *In silico* analysis together with immunofluorescence data and proteinase K accessibility assay suggest that leptospiral protein encoded by the gene LIC11834 is probably surface exposed. Moreover, the recombinant protein partially inhibited leptospiral adherence to immobilized laminin and PLG. We also show that this protein interacts with laminin. Thus, the recombinant protein was named Lsa33 (Leptospiral surface adhesin of 33KDa). The protein is also PLG - binding receptor, capable of generating plasmin in the presence of an activator. Although in a weak manner, the Lsa33 interact with C4bp suggesting a possible role in leptospiral immune evasion. We believe that this multifunctional protein has the potential to participate in the interaction of leptospires to hosts by mediating adhesion, may help the bacteria to escape the immune system and to disseminate through the host tissues.

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6.07 Characterization of three recombinant proteins of *Leptospira interrogans*

Fernandes LGV[1,2], Vieira ML[1,2], Kirchgatter K[3], Alves IJ[1], Morais ZM[4], Vasconcellos SA[4], Romero EC[5], Nascimento ALTO[1]

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Introduction: Leptospirosis is been considered an important re-emerging infectious disease caused by pathogenic *Leptospira* spp. The disease is transmitted from reservoir hosts to humans through water and soil contaminated with urine. At present, available vaccines based on inactivated whole cell of leptospires do not induce long-term protection against infection and do not provide cross-protective immunity against leptospiral serovars not included in the preparation. Surface exposed proteins are potential targets for inducing immune responses during infection and may also mediate the initial adhesion process to host cells. Furthermore, well conserved proteins are promising vaccine targets because they could induce cross-protective immunity against different serovars. **Objectives:** The goal of this work is to evaluate the immunological response of three recombinant proteins of *L. interrogans,* predicted to be outer membrane proteins (OMPs), in animal model and their capacity to adhere to serum proteins and extracellular matrix components. **Methods**: BALB/c mice were immunized subcutaneously three times, at 2-week interval, bled from the retro-orbital plexus and the antibody response evaluated by ELISA and Western blotting. The animals were sacrificed, and the splenocytes isolated for evaluation of lymphocyte proliferation and cytokine profiles. The attachment of these proteins to extracellular matrix and serum components was evaluated by ELISA. Reactivity with antibodies present in serum of confirmed leptospirosis samples, in both acute and convalescent phases, was also performed by ELISA. **Results and Discussion:** The recombinant proteins promoted high antibody levels in immunized mice.The protein rLIC10731 showed the highest proliferative rate. The cytokine profile evaluation for OmpL1 and rLIC10731 showed a significant increased level of IL-10, IFN-gamma and TNF-alpha. The OmpL1 protein showed a high reactivity with serum samples from confirmed leptospirosis patients, with 75% positivity at the convalescent phase, while 10% reactivity was detected at the early phase. All three recombinant proteins presented attachment to plasminogen and laminin, while OmpL1 also showed binding to plasma fibronectin, a data that suggests the involvement of these proteins in the leptospiral adhesion and infection process.

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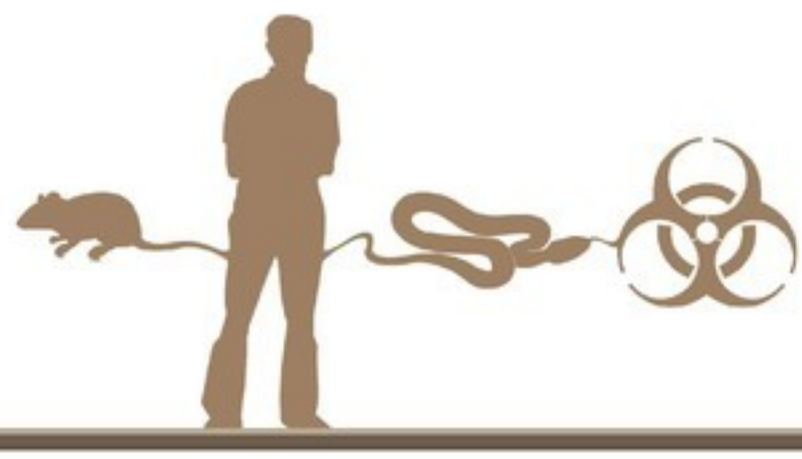

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6.08 Development of a chemically defined medium for capsular polysaccharide production by *Streptococcus pneumoniae* serotype 14

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Introduction: *S. pneumoniae* (pneumococcus) is a major cause of meningitis, pneumonia, and bacteremia, especially among young children and older adults. The capsular polysaccharides (PS) of pneumococci have been shown to be essential for their virulence and their production depends on regulation of their metabolic pathways. Sugars are the most preferred source of carbon and energy and several amino acids and vitamins should be supplied by the culture medium, since pneumococci are fastidious microorganisms with many metabolic deficiencies. **Objectives:** Evaluate the influence of amino acids and vitamins on the cell growth and PS14 production by *S. pneumoniae*. **Methods:** Fermentations were carried out in 0.5L bioreactors. A chemically defined culture medium (Van der Rjin & Kessler, 1980) was used as control experiment in triplicate (CDMc). The medium was modified by removing the following components: the amino acids asparagine, aspartic acid, phenylalanine, serine, alanine, threonine, tryptophan, lysine, and tyrosine (CDMA11); the vitamins folic acid, pyridoxamine, *p*-aminobenzoic acid, and β-NAD (CDMV6); and all aforementioned amino acids and vitamins (CDMM). In addition, the concentration of glutamine and glycine was doubled (CDMM+). The biomass was measured by optical density (OD) at 600nm (1.0 OD=0.366 g/L). The production of PS14 was measured by capture ELISA according to the methodology established in our laboratory. Sugar consumption and the production of organic acids were determined by HPLC. **Results and Discussion:** The biomass reached 0.81 g/L with CDMA11, while in CDMc it reached 1.30 g/L, the production of PS14 reached 115 mg/L with CDMA11, i. e., 77% of the amount produced in CDMc, 150 mg/L. The CDMV6 was equivalent to the CDMc, as CDMV6 productivity was 90% of complete medium (16.9 g cell/ L.h and 18.6 g cell/ L.h) and the biomass was 98% (1.30 g/L and 1.32 g/L). When both amino acids and vitamins were removed (CDMM), the biomass reached only 0.52 g/L, but the PS14 production (140 mg/L) was similar to PS14 of CDMc (150 mg/L). This indicates that the compounds taken off the medium affected cell growth but not PS14 production. The medium CDMM+ produced 137 mg/L of PS14 and 0.79 g/L biomass was reached, thus the increase of glycine and glutamine concentration raised the biomass but did not influence PS14 production. The vitamins and the amino acids mentioned above could be removed from the culture medium without drastic impact on production of PS14, thus composing a new culture medium for *S. pneumoniae* serotype 14, more economic and with less components. These findings could also contribute to elucidate the metabolic pathways involved in biomass and PS14 production.

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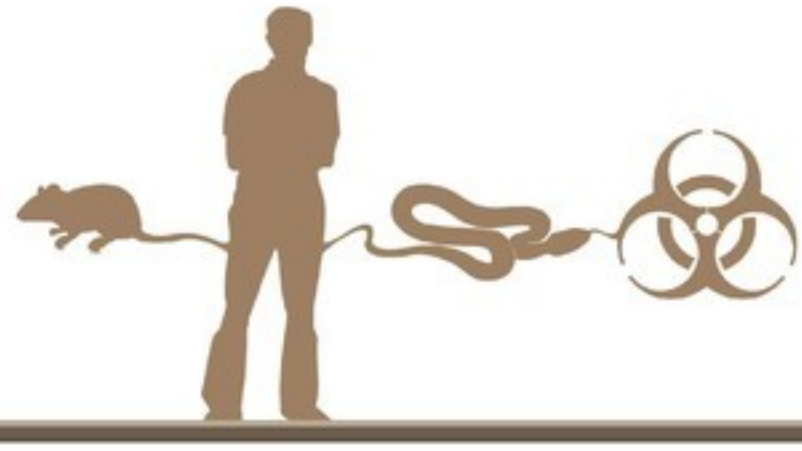

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6.09 Strategies for purification of pneumococcal surface protein A from clade 4 (PspA4)

Figueiredo DB, Ferri ALS, Marthos BV, Zanardo TR, Cabrera-Crespo J, Gonçalves VM
Laboratory of Bioprocess, Center of Biotechnology, Instituto Butantan, Brazil

Introduction: The use of pneumococcal proteins as carriers in conjugate vaccines could be an alternative to reduce the production costs and increase coverage. Pneumococcal surface protein A (PspA) has been shown promising results in animal essays and is conserved among pneumococcal strains. One of the most important steps in vaccine production process is the purification of its components, which must present a high degree of purity, and represents a major part of the production costs. **Objectives:** In this work we propose a purification process for PspA using precipitation aids: cationic detergent cetyltrimethylammonium bromide (CTAB) and cryoprecipitation, to increase purity before an anion-exchange chromatography. **Methods:** Cell concentration was measured by OD_{600nm} and protein by Lowry. The purity of PspA4 was determined by densitometry of SDS-PAGE bands. Cell pellet was diluted in 1mM NaH_2PO_4+2.5mM EDTA buffer with 1% Triton X-100 and 1mM PMSF. Cell disruption was done at 500bar for 15min by a continuous high pressure homogenizer, giving the Homogenate fraction. Homogenate was clarified by 2 methods: centrifugation alone or addition of 0.5% CTAB, giving respectively the Clarified and CTAB. An aliquot of Clarified fraction was precipitated with CTAB to obtain the Clean fraction. The Clarified, CTAB and Clean fractions were frozen/thaw and centrifuged to remove insolubles, resulting in cryoClarified, cryoCTAB and cryoClean fractions. The last two fractions with cationic detergent, CTAB, were pooled into a single fraction, poolCTAB. The fractions cryoClarified and poolCTAB were applied to a Q-Sepharose column, resulting in Q-Clarified and Q-poolCTAB fractions. **Results and Discussion:** Homogenate presented 43% of PspA4 purity. Clarified fraction displayed 51% recovery and 46% purity and CTAB fraction, 73% recovery and 83% purity. The use of CTAB in the Homogenate gave better results than in the Clarified fraction, since Clean fraction presented lower recovery and purity, 62% and 78% respectively. Cryoprecipitation was observed in all aliquots, increasing overall purity by 5% with no loss of PspA4. Q-Clarified fraction presented 28% recovery and 61% purity, while 33% of PspA4 was recovered in Q-poolCTAB fraction with 86% of purity. The increase of purity due to cryoprecipitation was advantageous as freezing for storage is a common step in the purification process. Precipitation with CTAB greatly increased the purity and recovery of PspA4 before anionic chromatography, thus resulting in higher purity in the chromatography, although the same recovery was observed in samples with and without CTAB precipitation. Further experiments should be done in order to enhance the recovery of the chromatography.

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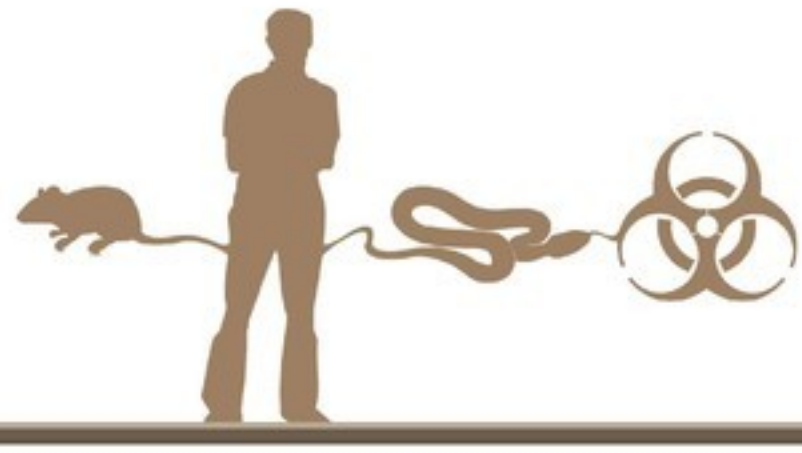

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6.10 Tetravalent dengue vaccine produced at Instituto Butantan

Frazatti-Gallina NM, Takinami VH, Miranda JRR, Rizzo R, Perrotti GG
Laboratório Piloto da Dengue, Instituto Butantan, Brazil

Introduction: Dengue is a common viral disease of humans. About 3.5 billion people are living in dengue-endemic countries. This disease is an important public health problem in Brazil mainly the hemorrhagic dengue. Nowadays the major challenge to researchers is to develop a good vaccine to prevent this disease. An attenuated tetravalent dengue vaccine lyophilized with 10 doses per vial was developed at Instituto Butantan. **Objectives:** To evaluate the stability of this vaccine after reconstitution and when this product (lyophilized form) was stored at 2-8°C. **Methods:** This vaccine was formulated with attenuated dengue virus 1, 2, 3 and 4 obtained from Vero cells infected with dengue virus strains from NIH (National Institutes of Health). Quality control tests were performed in this product to evaluate the safety, immunogenicity and stability. Three lots of this vaccine were used to evaluate the stability at 2-8°C (9 months) and after its reconstitution with diluent (8 hours). The vaccine reconstituted was stored at 2-8°C during 8 hours and samples were taken every hour to determine the titers of virus dengue 1, 2, 3 and 4 by PFA (Plate Forming Assay). In the stability study of the lyophilized form at 2-8°C, samples were taken every 3 months to determine the sterility, pH, residual moisture and virus titers. Virus titer of log10 2.5 (±0.5) PFU/dose was considered satisfactory. **Results and Discussion:** The geometric mean of the virus titers obtained in the samples of vaccine reconstituted were satisfactory for dengue virus 1, 2, 3 and 4 after six, five, four and five hours of storage at 2 to 8°C respectively. Considering the results found in dengue virus 3 the stability of this vaccine after reconstitution was determinate in 4 hours. The geometric mean of virus titers obtained in the lyophilized vaccine stored at 2-8°C during nine months were log10 3.2, 3.2 , 3.1 and 3.0 for dengue 1, 2, 3 and 4 respectively. The results found related to sterility, pH and residual moisture were satisfactory. The data found in this study showed that the tetravalent dengue vaccine has a good stability and will be used in Clinical Trials Phase I (2012).

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6.11 Stability study of pentavalent rotavirus vaccine using FFA test to determine the potency

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Introduction: Rotavirus is responsible for approximately 600,000 deaths per year in children under five years of age, mainly in Africa, Asia and Americas. This virus can cause a severe diarrhea in children. To prevent this disease, children less 5 years old are immunized with attenuated rotavirus vaccine. A new pentavalent attenuated rotavirus vaccine was developed at Instituto Butantan. This vaccine is composed of G1, G2, G3, G4 and G9 rotavirus serotypes and this product is presented in lyophyzed form with 4 doses per vial. To produce this vaccine, Vero cell cultures were infected with G1, G2, G3, G4 and G9 reassortant strains from NIH (National Institutes of Health). **Objectives:** To evaluate the stability of the pentavalent rotavirus vaccine when stored at 2-8 °C using the FFA test to determine the potency of each serotype present in this vaccine. **Methods:** Three lots of pentavalent rotavirus vaccine were used in this study. The lots IB-01/09, IB-02/09 and IB-04/09 with initial averages of potencies/serotypes of $10^{5.4}$ FFU/ml, $10^{6.0}$ FFU/ml and $10^{5.3}$ FFU/ml respectively were stored at 2-8 ºC during 40 months. Vials of each lot were taken each three months, and after reconstitution with citrate-phosphate diluents, samples of these vials were tested to determine the rotavirus titer by FFA (Fluorescent Focus Assay). The results of the tests were expressed in FFU/ml. This methodology determines the potency of each serotype. **Results and Discussion:** The averages of potencies/serotypes found in the vaccine samples after 40 months of storage at 2-8°C were $10^{5.0}$ FFU/ml, $10^{5.1}$ FFU/ml and $10^{5.1}$ FFU/ml for 01/09, 02/09 and 04/09 lots respectively. Considering that potency values $\geq 10^{5.0}$ FFU/ml for rotavirus vaccine are considered satisfactory to prevent severe diarrhea caused by rotavirus, the results found showed that the pentavalent rotavirus vaccine produced at Butantan has a good stability after 40 month of storage at 2-8°C.

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6.12 Expression of a complementation plasmid in a recombinant BCG-Pertussis after serial passages in IVM medium following good manufacture production

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Introduction: The live attenuated *Mycobacterium bovis* BCG (Bacille Calmette-Guérin) vaccine has been given to newborns as a safe vaccine to prevent severe and fatal tuberculosis and is an adjuvant treatment option for superficial bladder cancer. This disease is a common urologic cancer and the clinical course carries a broad spectrum of aggressiveness and risk. Low-grade, superficial bladder cancers have minimal risk of progression to death; however, high-grade non–muscle-invasive cancers frequently progress and muscle-invasive cancers are often lethal. Bladder cancer has the highest recurrence rate of any malignancy. Bacillus Calmette-Guérin (BCG) immunotherapy may be used for patients with recurrent disease or those at intermediate risk. Nascimento et al. constructed an rBCG strain expressing the genetically detoxified rBCG-S1PT and the immunotherapy with this strain resulted in bladder weight reduction. **Objectives:** In the present work we evaluated the expression of the complementation plasmid in rBCG-Pertussis during eight serial passages in IVM media following Good Manufacture Production (GMP). **Methods:** One or more clones of rBCG-Pertussis were used to inoculate eight subsequent cultures of sauton-potato medium. After 20 days pellet was expanded in eight consecutive passages in IVM pellicle medium. A different sample of each cultured batch was collected for S1PT expression analyses by immunoblotting. Total protein extracts were prepared and protein concentration in the culture lysates was determined. Approximately 30 µg of protein extracts were separated by gel electrophoresis (SDS-PAGE). The proteins were then electrotransferred onto a nitrocellulose membrane and the presence of S1PT was detected using a mouse polyclonal antiserum raised against detoxified PT. **Results and Discussion:** Western blotting showed that all samples from different passages expressed S1PT in comparable levels. These results indicate that the complementation plasmid in rBCG-Pertussis is stable after serial passages in IVM medium and maintains the S1PT expression levels. Thus, this complementation system is efficient for the production of the immunotherapeutic in GMP conditions. The rBCG-S1PT vaccine seed lots and certified vaccine lots will be produced for clinical trials.

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6.13 Purification of coagulation Factor VIII and Protein C by immobilized Co^{2+} affinity chromatography

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Introduction: Hemophilia A is an inherited bleeding disorder caused by deficiency of coagulation factor VIII (FVIII), while patients deficient in protein C (PC) are at risk of deep vein thrombosis. Use of liquid chromatography based methods to develop plasma fractionation processes is the aim of our group for many years. Recently we have been exploring the use of immobilized metal ion affinity chromatography (IMAC) in a systematic study with IMAC-Cu^{2+}, Zn^{2+}, Co^{2+}, Ni^{2+} and Fe^{3+} to separate FVIII from PC. Initially, plasma is applied to an anion exchange column, where FVIII and PC are separated from most of the plasma proteins and then in a IMAC column FVIII is well separated from PC. Here we present our most recent results on the purification with IMAC-Co^{2+}. **Objectives:** Evaluate the variation of pH and the concentrations of imidazole and NH_4Cl as desorbing methods in IMAC-Co^{2+} for the purification of FVIII and PC. **Methods:** Human plasma was directly applied to an anion-exchange ANX-Sepharose Fast Flow (FF) column. The eluate was applied to IMAC-Co^{2+} and 2 columns were tested (HisTrap HP 5 ml and IMAC Sepharose FF 10 ml). Analytical methods: Bradford, for protein content; chromogenic method, for FVIII and PC activities. **Results and Discussion:** In HisTrap HP 5 ml it was observed that 25 mM citrate buffer was capable of releasing the metal from the resin and therefore a 10 mM buffer was tested. In this concentration no leaking of the metal was observed. Using the ANX Sepharose FF eluate as the starting sample, PC was not adsorbed to the resin, while FVIII could be eluted in all the three methods. By increasing the imidazole concentration, FVIII eluted with 100 mM imidazole with purification factor of 8 times. This value is similar to that obtained with Cu^{2+} and Zn^{2+}. By decreasing the pH from 6.0 to 4.0, FVIII eluted with pH 5.0 with purification factor of 9 times. Using IMAC-Cu^{2+} proteins could not be eluted and with IMAC-Zn^{2+}, significant loss of activity was observed. By increasing of the concentration of NH_4Cl, FVIII was eluted mainly with 750 mM but the recovered protein was below the detection range of the Bradford method, indicating that the purification factor was high. Using IMAC-Cu^{2+} proteins could not be desorbed from the column with 1 M NH_4Cl and IMAC-Zn^{2+} proteins were desorbed with 1 M NH_4Cl. A 10 mL IMAC-Co^{2+} Sepharose FF column was packed to increase the amount of recovered proteins. Experiments with this column showed that the profile varying the desorption methods and the activity recoveries were similar to the observed with the HisTrap HP 5 ml column and the purification factor of FVIII was 11 times for all three desorption methods.

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6.14 Tuning gene expression by promoter engineering in mycobacteria

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Introduction: The Bacille Calmette-Guérin or BCG promotes an intense cellular and humoral immune response that is the baseline for using BCG as a live recombinant vector system to express antigens of different pathogens. This is achieved by transforming the bacillus with a plasmid capable of replicating in the mycobacterium containing the gene of interest under the control of a promoter. Since the first developments in recombinant BCG, the suitability of BCG to present a wide range of viral, bacterial and parasitic antigens has been confirmed. However, the level of gene expression obtained can be variable due to codon usage preferences among organisms, plasmid copy number or stability and most importantly the promoter strength. **Objectives:** The main objective is to generate promoters with different strengths through random mutagenesis of its genetic sequence. For this purpose, the fast growing *Mycobacterium smegmatis* was used as model. **Methods:** The mutation tool used was based on an error-prone PCR using modified dNTPs. The original pX promoter in the pXGFP plasmid was removed and replaced with the mutated versions of pX. This library of plasmids was then used to transform *M. smegmatis* and screened with respect to their fluorescence, which in turn represents the amount of GFP molecules expressed. The screening step was carried out in a fluorimeter using plates appropriate for both fluorescence and absorbance readings. Representative mutants from the range of fluorescence were cultured and analyzed using flow cytometry and qPCR and the plasmids purified for sequencing. **Results and Discussion:** We acquire 200 colony-forming unit (CFU) from the initial screening ranging between a 300-fold difference in fluorescence. The selected mutants showed specific changes in their promoter sequence, confirming the successful error-prone PCR strategy. Accompanying growth and fluorescence, we observed an increasing accumulation of fluorescent cells within a specific threshold of fluorescence. This peak agrees with the level of *gfp* mRNAs, indicating a transcriptional control rather translational. Once the heterologous expression level is an important parameter of host metabolism, carefully regulated to avoid wasting energy with unnecessary metabolites or prevent its growth if the molecule is toxic, and defining this condition and the promoters are perhaps the most important component of the regulatory control process, we proposed here a strategy to engineer promoters to control the gene expression that could minimize the disturbance in the regular metabolism of the mycobacteria, then improving its application as a recombinant delivery system.

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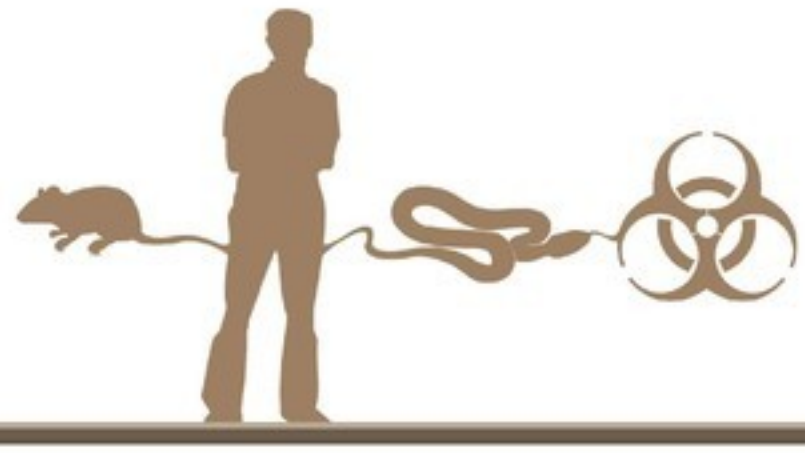

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6.15 Cellular growth in different bioreactors to Rabies virus production

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Introduction: The scaling up of virus production process involves different challenges, mainly when it is used cells of animal origin on a substrate. The growth of the animal cells in high densities depends on the beads and these cells present high susceptibility to the shear stress that occurs in the process performed in bioreactors. **Objectives:** The aim of this study was to evaluate the growth of Vero cells in the scale up process of rabies virus production in bioreactor. **Methods:** Two bioreactors were used in this study, one of 30 L (Bio Flow 4500, NBS) and other of 150 L (Bio Flo PRO Industrial, NBS). These bioreactors have different agitation systems: while the 30 L has a "Cell Lift Impeller", the industrial, one STR, has pitched blade impellers. This difference was important to select the velocity of agitation necessary to maintain the beads in suspension and to minimize the shear stress and bead collisions. Vero cells added to solid microcarriers, Cytodex 1 (2g/L), infected with PV rabies virus (MOI 0,02) were cultivated in serum-free medium VP SFM AGT in the two bioreactors. Seven cycles in each bioreactor type were done and the initial cellular concentration was 13-14 cell/microcarrier. Supernatants of these cultures were harvested on days 2 and 3 after the production cycle start. Samples of these cultures were taken every day during the production cycle to determine the cellular concentration. It was also studied the cellular loss in the first day after the cell inoculation to analyze the cell difficulty for spreading on the microcarriers. **Results and Discussion:** The averages of the cellular specific growth rate values found before the harvesting start were 0.025 h^{-1} and 0.023 h^{-1} in the industrial and 30 L bioreactors, respectively. The average percentages of cellular loss in the first day after cell inoculation were 37% (± 16%) in the industrial bioreactor and 52% (± 21%) in the bioreactor of 30 L. The analysis of these data showed that the spread and cellular growth were not affected by the blades of the impeller of industrial bioreactor. In conclusion, the growth expansion of Vero cell for rabies virus production in bioreactor of 150L was satisfactory since in this system the values of cellular loss and cellular specific growth rate were similar or better than the values obtained in the 30 L bioreactor.

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6.16 Optimization of inoculum preparation stage of BHK-21 cells for suspension culture in bioreactors

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Introduction: In industrial bioprocess, BHK-21 cell line has been widely used for viral vaccine production. The development of suspension culture systems, in most of the cases, allows high cell concentrations and volumetric yields, as well as facilitates the scale-up. Inoculum quality, culture medium composition, proper definition and control of bioreactor operational parameters are key elements for establishing a bioconversion process. **Objectives:** The aim of this work was to optimize the inoculum preparation stage of BHK-21 cells for suspension cultures in bioreactors. **Methods:** Two milliliter of non-adherent BHK-21 cell line ($2x10^6$ cell mL^{-1}) was thawed and added to 30 mL of culture medium (IMDM: 45.5%-DMEM, 45.5%-Pluronic (10% solution), 2%-Glutamine 4 mM solution, 2%-Inativated fetal bovine serum 5%) in T-flask (75 cm^2, in vertical position). After 96 hours in culture ($8x10^5$ cell mL^{-1}), this cell suspension was used as inoculum in T-flasks (25 cm^2, vertical position) to study the influence of liquid column height (5, 10 and 15 mm) on maximum cell concentration (X_{max}), maximum specific growth rate (μ_{max}) and cell viability for static culture. Each condition was performed in triplicate and evaluated during 142 hours. In spinner flasks culture stage (100 mL, 25 rpm), a factorial multilevel experimental design was performed to assess the significance of working volume (30, 50, 65 mL) and inoculum volume percentage (10 and 30%) related to working volume on the same response variables of the previous stage. All the experiments were performed at 37 °C and 5% CO_2 atmosphere. **Results and Discussion:** Five millimeter of liquid column height showed X_{max} ($2.0 \pm 0.1x10^6$ cell mL^{-1}), 1.42-fold higher than the X_{max} obtained for 10 mm height ($1.4 \pm 0.1x10^6$ cell mL^{-1}). Therefore, it was possible to save 30% of culture medium for this stage. After 96 hours, cell viability for the three conditions under study was in 98.2-99.6% range. In spinners flasks, the best working volume, considering high cell density, was 50 mL ($p=0.004$); inoculum volume percentage ($p=0.085$) did not show statistical influence on X_{max}. In these conditions, the ranges for μ_{max} and X_{max} were 0.85-0.93 day^{-1} and 4.1-$4.2x10^6$ cell mL^{-1}, whereas cell viability was higher than 90% (96 hours cultures). Based on these results, a protocol for BHK-21 inoculum preparation was defined for a future transient expression, with optimization of time and supplies.

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6.17 Methodology based on UV-Vis spectroscopy and partial least squares for metabolism and growth monitoring of BHK-21 cell cultures in bioreactors

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Introduction: Biomedia characterizations or even, determinations of certain compounds with specific analytical techniques are usually costly. The global direct methods were developed to overcome this trouble. They are a combination of a *hard* part, for sensing and a *soft* part, for data treatment. Spectroscopic methods associated to multivariate data analysis have been used for bioprocess monitoring. Nevertheless, UV-Vis spectroscopy has been poor utilized for this purpose, because of spectral and operational restrictions. Valuable information from these spectra can be extracted with chemometric methods. **Objectives:** The aim of this work was to adjust partial least squares (PLS) models for predicting key nutrients and metabolites as well as viable cell concentrations for BHK-21 suspension cell culture in bioreactors based on off-line UV-Vis spectra. **Methods**: Batch experiments (37°C, 80 rpm,) with BHK-21 cells were performed in Celligen (2L, 400 mL/min volumetric gas flow) and Bioflo 110 (1L, 200 mL/min) bioreactors at dissolved oxygen concentrations from 10 to 70 % air saturation. In some batches, a $NaHCO_3$ solution (8 % m/v) was added to control pH (7.2), for the remaining batches, pH was controlled until it was possible with gas mixtures (Air, N_2, CO_2, O_2). Eight or nine samples were collected for each batch. Glutamine, glutamate, glucose and lactate were quantified by enzymatic methods. Cell concentration was determined by hemocytometer. Spectral analyses of diluted samples (10-fold) without cells from 280 to 800 nm were done. PLS modeling was performed in SIMCA 13 demo version software. **Results and Discussion:** Three spectral peaks were detected, two associated to pH indicator (Phenol red, 430 and 560 nm) and another one in protein absorption range (286 nm). Monitored variables were mainly dependent to the absorbances changes around 430 and 560 nm. The maximum prediction errors for glutamine (0.31 mM), glutamate (0.05 mM), glucose (2.21 mM) and lactate (3.08 mM) concentration were in agreement with others previously reported, using other methodologies. However, cell concentration models showed prediction error higher than 15%. Probably, this may solve increasing the training data set. This work could be useful to define fed-batch feeding schemes and to establish the principles for developing an at-line sensor.

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6.18 Prophylactic HPV vaccine for broad spectrum protection

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Introduction: Cervical cancer is one of the leading causes of cancer death in women worldwide. Human papillomavirus (HPV) is associated with genital warts and several types of cancer, including cervical cancer, caused mainly by high-risk HPV16 and 18. The icosahedral capsid of HPV is composed of two structural proteins, L1 and L2. HPV16 VLPs (virus-like particles) L1 has been utilized in the vaccine development due to their capacity to induce high immunological response. L2 can induce a lower titer of antibodies, although to a wide range of divergent papillomavirus. **Objectives:** To design a prophylactic vaccine based on the antigens HPV16 L1L2 structured capsid proteins, protective against viral types related to a wide range of HPV-associated human cancers. **Methods:** Cell cultures of HEK293T were transfected with DNA expression vectors encoding for L1 and L2 proteins of HPV16. The proteins expression was detected by western blotting, confocal microscopy and electron microscopy. For immunolabeling we used anti-HPV16 L1 and anti-HPV16 L2 primary antibodies and secondary antibodies conjugated with fluorochromes or colloidal gold particles. Confocal microscopy was also used in kinetic curves for establishing the most appropriate moment to harvest transfected cells for protein purification. **Results and Discussion:** HPV16 capsid proteins were detected by cellular and molecular procedures in nucleus and cytoplasm of transfected cells. We successfully established an efficient system of HPV16 L1 and L2 proteins production for the development of an HPV prophylactic vaccine. Purification assays are being carried out and the vaccine will be tested in mice to evaluate its capacity to induce immune responses to HPV16 and other types. This prototype vaccine could embody public health programs, especially in developing countries.

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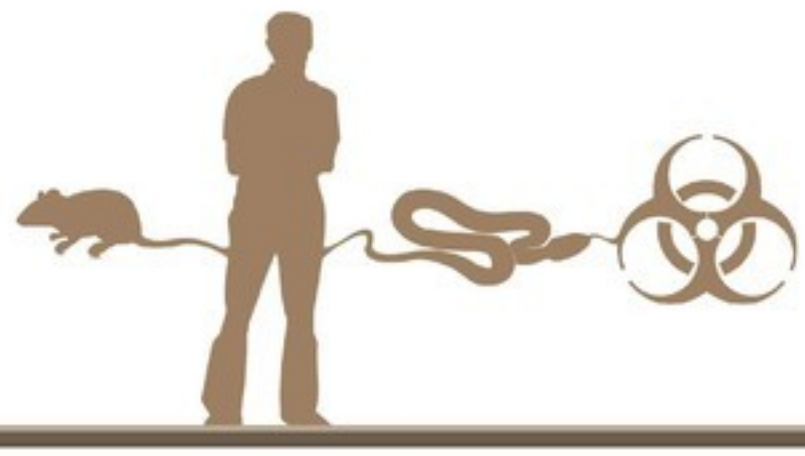

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6.19 Optimization of the capsular polysaccharide production in *Streptococcus pneumoniae* serotype 1 cultivation: an approach for process development

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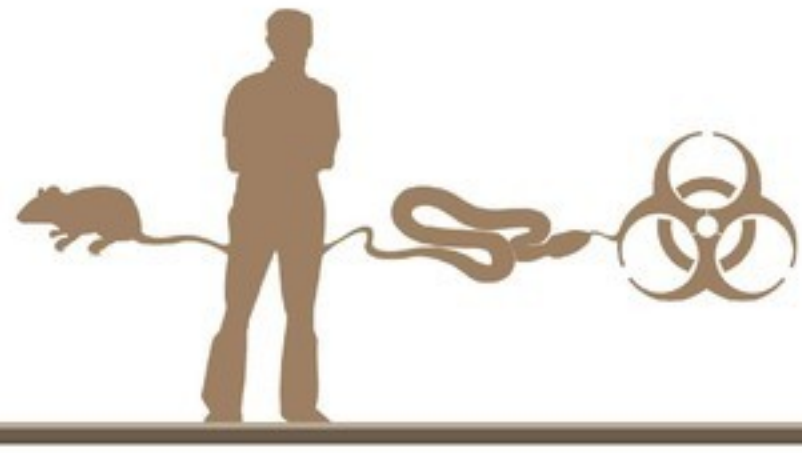
Introduction: *Streptococcus pneumoniae* is a human pathogen transmitted by aerosols. Pneumococcal vaccines are the main strategy against this pathogen and the capsular polysaccharide (PS) is the major pneumococcal antigen. Nowadays there is a lack of information on polysaccharide production process in the literature, probably because this know-how is an industrial secret of pharmaceutical companies. Therefore, producers such as Instituto Butantan have to establish their own technology to produce pneumococcal vaccines. **Objectives:** The aim of this work was to develop all steps of pneumococcal cultivation process to produce PS of serotype 1 (PS1) and to reduce the cost of culture medium. **Methods:** Standard culture medium (SCM) was composed of 30.0g/L peptone (Casaminoacids, Phytone, or Soytone), 20.0g/L yeast extract (YE), 0.10 g/L Asn, 0.650 g/L Gln, 20.0 g/L glucose, 0.010 g/L choline, salts and phosphates. Biomass was measured by optical density at 600nm (1.0 OD=0.39 g/L of dry weight). PS1 production was determined by MHDS method. Residual glucose and organic acids production were detected by HPLC. For screening, 9 strains of *S. pneumoniae* serotype 1 were grown in flasks using SCM with Casaminoacids, the two best strains were grown in 5L-reactor and the best PS1 producer was selected. This strain was cultivated to define the peptone of the medium: Casaminoacids, Phytone, or Soytone, in duplicate reactor experiments. In order to optimize the medium, 12 fermentations were carried out following a design of experiments (DoE) with four factors (peptone, YE, Asn, and Gln). Finally, 2 cultivation strategies were evaluated: batch and fed-batch, using the culture medium composition based on the results of DoE. The feeding medium for fed-batch was concentrated 4-fold in glucose, peptone, and YE, all other compounds remained in the same concentration. **Results and Discussion:** The strain ST 595/03 produced 90mg/L of PS1 and was selected as the best producer. Phytone was chosen due to higher PS1 production than other peptones, reaching 300mg/L, 33% higher than Soytone and 89% higher than Casaminoacids. DoE results indicated a new medium formulation with 15g/L Phytone, 2g/L YE and without Asn and Gln. The highest PS1 production was reached in fed-batch, 372mg/L, 144% higher than batch. The different tools used for process development in this work allowed us to increase the PS1 production 4.1-fold and reduce the cost of medium by saving 68% of the YE amount added, removing amino acids and replacing Casaminoacids by Phytone, which is 2.5 times cheaper and free of animal compounds. In addition, this successful step by step process development can be applied to other serotypes.

Supported by: FAPESP and CAPES



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6.20 Production of hemoderivatives at Instituto Butantan – The pilot plant and production of IVIG

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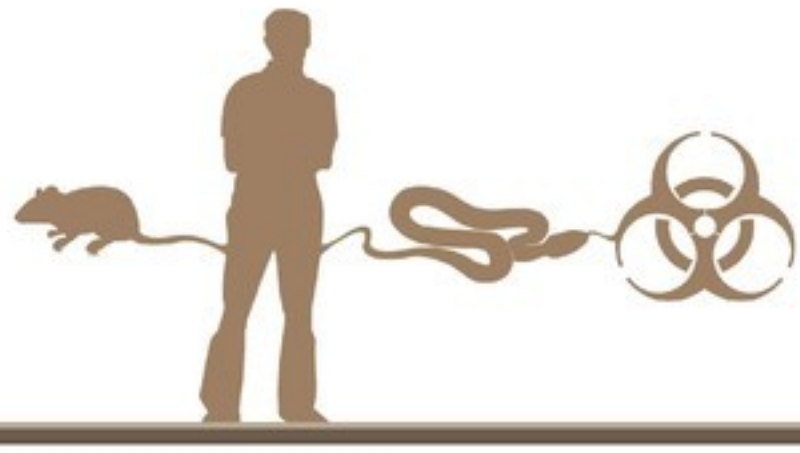
Introduction: An industrial facility for plasma fractionation is under construction at the Instituto Butantan with the support of the Secretary of Health of SP. The process of fractionation, based mainly in chromatographies, was designed by GE Healthcare's R&D for production of albumin, immunoglobulin (IVIG) and coagulation factors VIII and IX in batches of 1250 L plasma. **Objectives:** In parallel to the construction of the industrial facility, a pilot plant was built with the objective to test each step of the process and establish the scaling up parameters in 25 liters of plasma. It was also meant to start training the personnel in techniques that will be used for plasma fractionation, such as ultrafiltration, chromatography and continuous centrifugation. The pilot runs would also be used to establish assays for in process control and quality control of final products. **Methods:** To learn the process, several runs producing IgG were carried out in the pilot plant, as IVIG is meant to be the first hemoderivative obtained in the industrial facility. The production of IVIG process consists in 6 chromatographies, three ultrafiltration and one continuous centrifugation steps. The columns were packed, hollow fibers systems for protein concentration and continuous centrifugation were assembled and tested. Collected fractions were analyzed by SDS-PAGE and western blot. **Results and Discussion:** Previously, four partial runs, in which two chromatographic steps were skipped, were performed for IVIG separation and then our team processed two complete pilot runs, as designed for the industrial production. The chromatograms were analyzed showing a reproducible process. Many process parameters were collected for the scaling up, including the ones to determine the size of the continuous centrifuge necessary for the industrial facility. Analysis by SDS-PAGE showed that about 3.2 g of IgG is recovered per liter of plasma as final product. The presence of factor XI in IVIG concentrates is a concern and is being investigated by western blot in the final products of the different runs. Some of the chromatographic fractions were identified as initial material for process development of potential new products. The proposed goals for this non GMP pilot plant were achieved, with the exception of the quality control tests, which are being under development. The pilot plant allowed performing successfully manual processes in 12.5 – 25 L scale. A full process could be envisaged considering the need of automatization for larger scales. The Research and Development team from GE healthcare participated in the runs performed at the hemoderivative pilot facility.

Supported by: Fundação Butantan, CAPES



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6.21 Characterization of a novel protein antiviral from *Lonomia obliqua* using bioinformatics tools and activity analysis by real time

Mazur TR[1], Mazzoni MKF[1], Carmo ACV[1], Giovanni DNS[1], Yamasaki LHT[2], Figueiredo CA[3], Oliveira M I[3], Santos FCP[3], Curti S P[4], Martins LM[1], Araujo RL[1], Tonelotto M[1], Rahal P[2], Mendonça RZ[1]
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Introduction: The control of viruses is of great interest to the public health area. Several studies have been conducted that show the presence of pharmacologically active substances in the hemolymph of insects. Recently we have demonstrated the existence of a potent antiviral in the hemolymph of *Lonomia obliqua* caterpillar (rAVLO). This protein was able to reduce at 10^6 times the replication of herpes virus and in 10,000 fold the rubella virus. **Objectives:** The aim of this work was the characterization of an antiviral protein from *lonomia obliqua* using bioinformatics tools and activity analysis by real time. **Methods:** RT-PCR was used to determine viral RNA present in cultures treated and no treated with rAVLO protein. These cultures were infected with Herpes and Rubella virus. After determination of antiviral action of the recombinant protein (rAVLO), the sequence of this protein was determined by bioinformatics tools. **Results and Discussion:** RT-PCR used to determine viral RNA present in treated and non-treated infected cells with rAVLO showed a virus reduction of 10^6 times to herpes virus and in 10^4 fold to rubella virus. The analysis of this protein by bioinformatics suggests that this protein is globular, secreted with a signal peptide which is cleaved between amino acids 16 and 17. The studies also allow us to infer that this antiviral protein has the ability to bind to MHC class I. It was found that there are several protein binding sites on the weak and strong bases with various HLA. The bioinformatic analysis also shows a strong presence of α-helices in the N-terminal region and allowed to classify the antiviral protein as α/β type of structure, as we detected the presence of more than 30% α-helix and 20 % of β-sheet found separately along the protein chain. In the BLAST sequence analysis of cDNA antiviral protein, no sequence similarity was found in Genbank, suggesting that it is from a novel protein family. It can be inferred by an analysis of this region that the possible antigenicity region would be between the 70-110 amino acids, showing high accessibility. This high antigenic region on the surface can be a possible region to interaction with other proteins.

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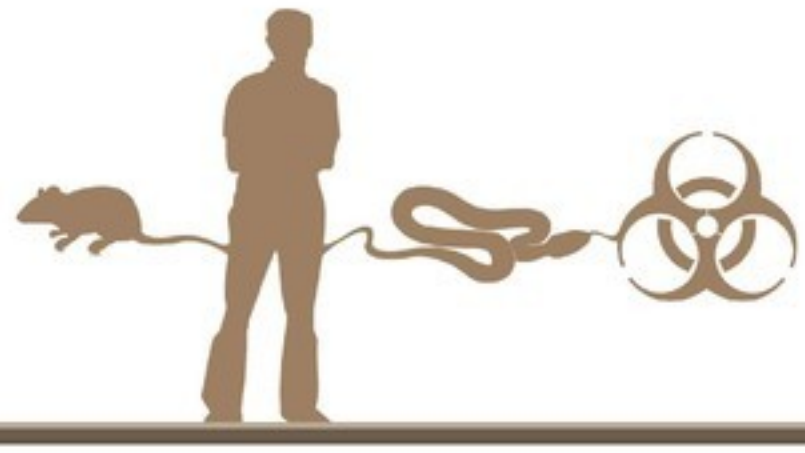

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6.22 Production of a recombinant protein from *Lonomia obliqua* caterpillar, with antiviral activity, in Baculovirus/SF-9 cells system

Mazzoni MKF[1], Mazur TR[1], Carmo, ACV[1], Giovanni DNS[1], Soares JS[1], Araujo RL[1], Martins LM[1], Veiga ABG[2], Moraes RHP[1], Mendonça RZ[1]
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Introduction: The viral infection control is of great interest in Public Health. Several studies have shown the presence of active compounds in the hemolymph of arthropods, some of which are of interest for the development of new pharmacological drugs. Recently we have demonstrated the existence of a potent antiviral in hemolymph of *Lonomia obliqua* caterpillar. This purified protein reduced virus production ($TCID_{50}$ ml^{-1}) more than 157 fold (from $3.3\pm1.25x10^{7}$ to $2.1\pm1.5x10^{5}$) to measles virus, 61 fold to polio virus ($2.8\pm1{,}08x10^{9}$ to $4.58\pm1.42x10^{7}$) and 64 fold to H1N1 influenza virus. **Objectives:** This study aims to build recombinants bacmids containing sequences encoding this antiviral protein in baculovirus/SF-9 cell system and and test the antiviral activity of recombinant protein. **Methods:** To synthesize cDNA, RNA of *L. obliqua* was extracted and used in polymerase chain reactions using reverse transcriptase polymerase (RT-PCR) with specific primers for the antiviral protein, based on the sequence of the cDNA libraries of *L. obliqua* tegument and spicules. Restriction sites were inserted in the cDNA for connection to the donor plasmid pFastBac1™ (Invitrogen). The recombinant plasmid was selected in *Escherichia coli* DH5α and subsequently used in the transformation of DH10Bac *E. coli*, to obtain the recombinant bacmids. This bacmid, containing the sequence of a protein with antiviral activity was used for expression of this protein in baculovirus/SF-9 cells system. **Results and Discussion:** In order to investigate the antiviral effects on picornavirus (EMC encephalomiocardite), whole hemolymph and recombinant protein (1% v/v) were added to the L929 cells cultivated on 96-well plates, 1 hour before infection with 100 TCID50 of virus. Samples of the cell cultures were collected daily and analyzed to determine the percentage of cells with cytopathic effect (*CPE*). The recombinant protein was able to block the replication of 100 TCID50 of picornavirus (EMC), showing that the recombinant antiviral protein remains fully active.

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6.23 *In silico* analysis of E6 BPV-1 recombinant protein

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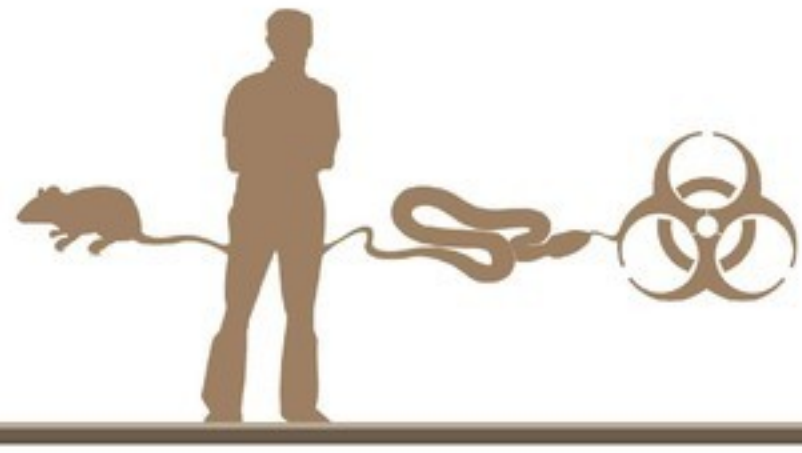
Introduction: Considering human and animal health, the papillomaviruses (PVs) are described as infectious and contagious agents. These viruses induce the formation of benign lesions (papillomas) in the skin and/or mucous membranes which may eventually become malignant. Among BPVs described, BPV-1 causes fibropapillomas in its natural host (cattle) and sarcoids in equines. The BPV-1 E6 gene encodes a 137 amino acids protein that acts as a transcriptional activator, p53 protein ligand, affecting also telomere activity. Thus, E6 is potentially oncogenic which makes it a target for the development of therapeutic strategies. The development of biotechnological E6 associated products depends not only on obtaining the recombinant protein, but also on its structural and functional analysis for better and reliable results. **Objectives:** Amplify, cloning and sequencing of BPV-1 E6 gene in order that both nucleotide and predicted amino acid sequence can be analyzed in various aspects such as structure and immunogenicity. **Methods:** After amplification of the E6 gene of BPV-1 and subcloning into pET28a bacterial expression vector, plasmids were sequenced. The nucleotide sequence was translated into amino acids and both were analyzed, using as a reference the BPV-1 E6 sequences deposited in GenBank (accession number NC_001522 and Protein id NP_56737-1). The sequences were aligned and their identity matrix was calculated (ncbi.nlm.nih.gov using Blast and Cobalt tools; BioEdit software). A bioinformatics analysis was made on the conserved regions of E6 proteins of different viral types, regarding both structure and immunogenicity (cunsurf.tau.ac.il and bioinformatics.org/JaMBW software). The prediction of 3D structures was also performed (swissmodel.expasy.org software). The E6-1 recombinant protein was expressed and purified allowing subsequent more detailed studies. **Results and Discussion**: Analysis of the E6-1 gene and protein sequences identified four mutations, half of which were silent mutations. Protein domains showed that these mutations occurred at motif points less conserved, that probably not change the overall structure and function of the recombinant E6 protein. Further generated graphics reveal the E6 immunogenic regions. E6-1 recombinant protein was successfully expressed and purified, allowing new studies such as crystallography and protein sequencing. Briefly, the detailed study of recombinant proteins is essential to obtain safe and quality therapeutic bioproducts.

Supported by: CAPES, CNPq, FAPESP and MCT



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6.24 Comparison of the expression systems pnirB and soxRS using eGFP as reporter

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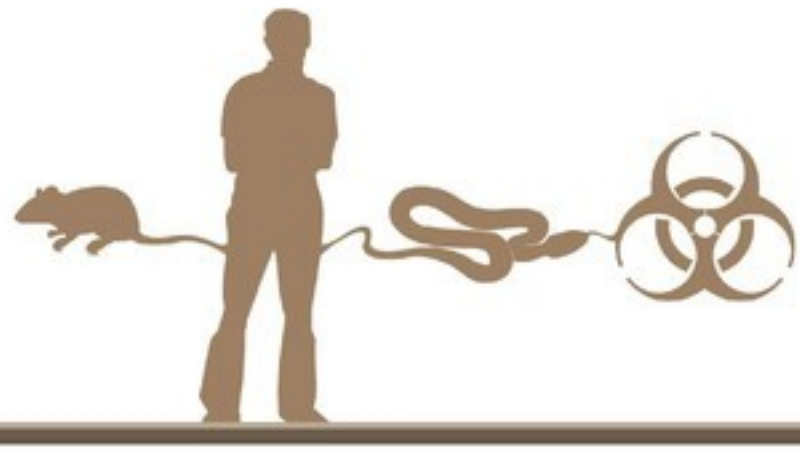
Centro de Biotecnologia, Instituto Butantan, São Paulo, Brasil.

Introduction: Attenuated *Salmonella* strains are being studied as live vaccines carrying heterologous antigens. Interesting characteristics of the system are: salmonellas can be delivered orally, able to invade the organisms, posses a tropism for macrophage, an antigen presenting cell, can deliver multiple antigens, can elicit a more complete immune response compared to immunization with purified antigens acting as adjuvant. However, the effectiveness as vaccine depends on the ability to express the heterologous proteins in enough amount and proper time. During infection, the macrophages impose a respiratory burst to the invasive salmonellas, generating superoxide, nitric oxide and other types of oxidative stress. In response, an antioxidant defense is activated in salmonella. Among the resposes, the soxRS regulon controls the expression of several antioxidant proteins. We propose to use the control systems of the soxRS regulon as promoter controlling the expession of heterologous proteins. This way, the expression of antigens of interest would be directly activated in the macrophages. This promoter system can be activated *in vitro* by adding paraquat to the bacterial cultures. Several expression promoters are described in the literature. One largely used is the nitrate reductase promoter (pnirB), which is activated in conditions of low oxygen tension, such as that found in the mammalian environment. **Objectives:** The aim of this work was to compare the efficiency of the promoter systems soxRS and pnirB controlling the expression of recombinant proteins in salmonellas in vivo. For this purpose, the expression of green fluorescent protein (eGFP) was used as reporter. **Methods:** Two vectors were constructed pAEsox and PAEnir and the gene of eGFP was cloned to transform the attenuated salmonella SL3261. The expression of the recombinant protein in SL3261pAEsoxGFP was tested in vitro using paraquat and in SL3261- pAEnirGFP was tested removing oxygen by completely filling the culture in a glass tube and sealling to avoid any oxygen input. Samples of the culture were collected at different times to measure the OD_{600nm} and the intensity of the fluorescence. A fluorometer was used light exciting 488 nm and fluorescence measured at 506 nm. Values of fluorescence were normalized by the OD. **Results and Discussion:** The *in vitro* experiments showed that the promoter system soxRS was effective, for controlling the expression and leading to higher intensity than pNir. The anaerobioses imposed to activate pNir, totally inhibited the growth of SL3261pAEnirGFP. Besides, fluorescence was high during aerobic growth, indicating no control of expression. The SL3261pAEsoxGFP is being tested *in vivo* to demonstrate the expression in the macrophages.

Supported by: Fundação Butantan, CNPq PIBIT



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6.25 Cloning, expression and purification of the Lsa66 protein fragments of *Leptospira interrogans*

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Introduction: *Leptospira interrogans* is the etiological agent of leptospirosis, a zoonotic disease of human and veterinary concern. The identification of novel proteins that mediate host-pathogen interactions is important for understanding on the molecular mechanisms of leptospiral pathogenesis and could also facilitate the identification of novel vaccine candidates. Lsa66, the ompA-like leptospiral protein, is capable to bind laminin, plasma fibronectin and plasminogen. Lsa66 was the first leptospiral protein reported to interact with ECM and PLG. **Objectives:** The aim of this study is to identify the immunodominant and the ECM/PLG binding domain(s) of Lsa66. **Methods:** The fragments corresponding to the N-terminal, intermediate and C-terminal of the LIC10258 gene were amplified by the PCR methodology from *L. interrogans* serovar Copenhageni genomic DNA using the complementary sequence primers. The PCR products obtained for each corresponding fragment were cloned into pGEM-T easy vector and subcloned into the pAE expression vector at the BamHI/NcoI cloning sites. All cloned sequences were confirmed by DNA sequencing with an ABI 3100 automatic sequencer. The pAE constructs were employed to transform BL21 SI, BL21 Star pLys, BL21 DE3 and C43 *E. coli* strains. Protein expression was analyzed in several conditions, including IPTG or NaCl concentrations and protein expression induction at different temperatures. **Results and Discussion:** Recombinant fragments expression were analyzed through 12-15% SDS-PAGE in which the expected protein bands of ~ 22.55, 36.9 and 20.3 kDa, corresponding to N-terminal/LIC10258, intermediate/LIC10258 and C-terminal/LIC10258, respectively, were visualized by Coomassie blue staining. All recombinant fragments were expressed in insoluble form as inclusion bodies and solubilized in 8 M urea. The purification of the proteins through metal-chelating chromatography rendered, in each case, a major protein band, suitable for biological assays.

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6.26 Establishment of defined medium for growing *Haemophilus influenzae* type b

Paiva PR[1], Cintra FO[1], Portas VB[1], Auada AVV[2], Lebrun I[2], Takagi M[1]
[1]Laboratório de Fermentação, Centro de Biotecnologia, Instituto Butantan, Brazil; [2]Laboratorio de Bioquimica, Instituto Butantan, Brazil

Introduction: *Haemophilus influenzae* type b (Hib) is a Gram negative pathogenic bacterium, responsible for causing pneumonia and meningitis in infants and elderly. Extracellular polysaccharide composed of poly-ribosyl-ribitol-phosphate (PRP) is the main virulence factor and the specific antigen used in the Hib vaccine. *H. influenzae* is fastidious and requires enriched medium to grow; as animal based medium is not recommended for vaccine production due to the risk caused by prions, medium of vegetal origin has been used for Hib growth and PRP production when supplemented by hemin and nicotinamide adenine dinucleotide (NAD). Defined medium is not used for PRP production due to low cell density; however, once improved it would be appropriate to study Hib`s metabolism. **Objectives:** In this work an elaboration of defined medium for Hib growth was proposed in order to understand its metabolism. **Methods:** Experiments were carried out in shake flasks at 36°C and 200 RPM using RPMI 1640 medium (cat. 61870036) supplemented with sodium pyruvate (cat.11360070), NAD (15mg/L), hemin (30mg/L), uracil (87mg/L), glucose (5000 mg/L) and modified as follows: 1) without inosine and hypoxanthine; 2) with hypoxanthine (control); 3) medium 2 plus glutamic acid; 4) medium 2 plus NH_4Cl (as N source). The inoculum was initially activated by static incubation at 37°C in a chamber jar with low oxygen tension for 6h and then incubated overnight at 37°C, 200 RPM. An amount of this inoculum was transferred to each flask in order to reach OD_{540nm} of 0.1 and to start the cellular growth kinetic, which was followed by reading the OD_{540nm} every hour. **Results and Discussion:** The growth profile in the presence of glutamic acid achieved an OD_{540nm} of 2.16 followed by the medium 4 with OD_{540nm} of 1.6, both overcoming the control medium with OD_{540nm} of 1.4. No growth was observed when inosine and hypoxanthine were absent. Hib is deficient in three enzymes of the citric acid cicle, which makes this microorganism unable to grow properly. Glutamic acid has an important role in the formation of some amino acids essential for cellular growth. Inosine is a precursor for nucleic acid synthesis and, according to Hib's genome model, the experimental data confirms that hypoxanthine replaces inosine. In conclusion, the defined medium based on RPMI and enriched with glutamic acid and NH_4Cl is feasible to study Hib´s metabolism and the improvement of this medium could be used in the future for PRP production.

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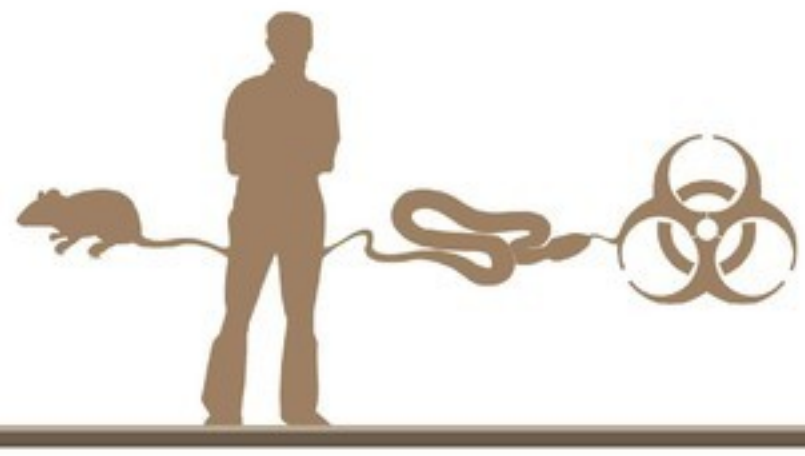

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6.27 Effect of different detergents in the purification of capsular polysaccharide produced by *Haemophilus influenzae* type b

Portas VB, Cintra FO, Paiva PR, Takagi M, Cabrera-Crespo J
Laboratório de Bioprocessos, Centro de Biotecnologia, Instituto Butantan – Brazil

Introduction: *H.influenzae* b (Hib) is an encapsulated Gram negative pathogenic bacterium responsible for pneumonia and meningitis in children. The capsular polysaccharide (PRP) is the main virulence factor of Hib and is used as the antigen in combination with a protein for vaccines. The classical polysaccharide purification process includes precipitations with organic solvents, phenol extraction to remove proteins (Prot) and nucleic acids (NA), centrifugation/ultracentrifugation, and anionic detergent as DOC for removal of lipopolysaccharides. The relative purity based on proteins (RP_{Prt}) and nucleic acids (RP_{NA}) should be ≥100 (RP= amount of PRP/amount of Prt or NA) as required by the World Health Organization. **Objectives:** The aim of this work was to use different detergents and evaluate their effects on PRP purification. **Methods:** The inactivated culture of Hib was centrifuged and the supernatant was concentrated by tangential ultrafiltration (100kDa cut-off) and fractions of this concentrate were used to test the different detergents with ethanol 30%: DOC and SDS (anionic), Triton X-100 and Tween-20 (neutral). To the concentrated PRP fraction were added the detergents and ethanol to achieve a final concentration of 0.3 and 30%, respectively. The samples were allowed to stand for ~16h and then subjected to centrifugation to remove the insoluble material. The supernatant was subjected to a second precipitation step with ethanol at 80%. The precipitate containing PRP, separated by centrifugation, was dissolved in purified water and subjected to two further precipitations with 80% ethanol and the last water soluble PRP was submitted to centrifugation to remove insoluble impurities. In each step PRP, Prot and NA concentrations, were determined to evaluate the purification efficiency considering PRP recovery (%), RP_{Prt} and RP_{NA}. **Results and Discussion:** The average recovery of PRP was 46%~50%, for all detergents, indicating no significant loss of PRP. However the RP_{prt} was 7.8, 60.6, 10.5 and 10.6 and RP_{NA} was 28.2, 392.2, 22.3 and 31.5 and SDS showed the best results. It was also found that the best SDS concentration for the impurities removal was 0.5%, with RP_{prt} = 87.1, RP_{NA}= 651.8 with 58.5% PRP recovery. Purity of PRP with respect to NA has reached the required purity, while proteins require a further polishing step. In conclusion, addition of SDS to the ethanol precipitations steps improves the purification process. SDS is a detergent with strong denaturant activities on Prt, NA and lipid membranes, what may have facilitated the action of the ethanol precipitation.

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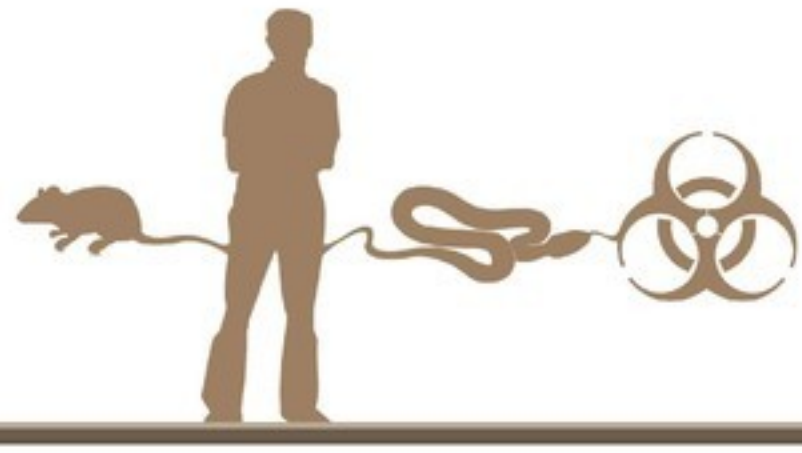

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6.28 Monophosphoryl Lipid A as adjuvant in acellular pertussis vaccine

Prestes AFRO[1], Sakauchi MA[2], Kubrusly FS[1], Iourtov D[1], Raw I[1], Dias WO[1]
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Introduction: The monophosphoryl lipid A (MPL A) is a detoxified derivative of lipopolysaccharide (LPS) present in the cell wall of gram-negative bacteria such as *Bordetella pertussis*. The MPL A has the immunostimulatory activity of LPS, without their reactogenicity. The Butantan Institut has developed a procedure for the production of MPL A from the culture of *B. pertussis*, used as adjuvant in vaccine formulations, by removing the LPS from the whole cell pertussis vaccine (wP), resulting in a less reactogenic and as well as effective whole cell pertussis vaccine (P_{low}) and MPL A as a sub product. In the same line of production is also obtained an acellular pertussis vaccine (aP) from the culture supernatant. **Objectives:** The objective of this work was to evaluate the immune response induced by the aP combined with diphtheria and tetanus toxoids (DTaP), using alum (AL) or MPL A as adjuvant, in mice. **Methods:** Humoral immune response induced DTaP, DTaP + AL and DTaP + MPLA was measured by ELISA, against detoxified pertussis toxin (dPT), or diphteria and tetanus toxoids (DT and TT). IFNg production was evaluated in culture supernatants of splenocytes from immunized mice, after "*in vitro*" stimulation with dPT, or DT and TT. **Results and Discussion:** It was obtained high IgG antibodies titers anti-pertussis, anti-diphtheria and anti-tetanus toxin in mice immunized with DTaP vaccine, with or without AL or MPLA as adjuvants. Moreover, it was observed high and similar titers of IgG1 and IgG2a anti-pertussis toxin, significantly higher than the obtained for the control groups, but no statistical difference was found between trhe experimental groups. It were detected the production of IFN-γ by the splenocytes of the mice immunized with DTaP + MPLA, 72 hours after *in vitro* stimulation with dPT, DT and TT. The DTaP, with or without the adjuvants induced similar IgG antibody response in immunized mice, as well as similar IgG1 and IgG2a antibody titers, suggesting Th1/Th2 balanced response. The IFN-γ production in immunized animals with DTaP+MPLA suggests tendency to Th1 immune response, which will be better investigated by our group, since the immunemodulation to Th1 response would simulate the protection elicited by native infection with *B. pertussis*.

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6.29 Evaluation of GFP expression using Semiliki Forest Virus expression system

Puglia ALP[1], Pereira CA[1], Rezende AG[1], Silva FG[1], Boldorini VL[1], Novaski IA[2], Jorge SAC[1], Astray RM[1]

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Introduction: Semliki Forest Virus (SFV) has been studied for recombinant protein production in infected mammalian cells, for therapeutics or as a vaccine vector. Expressing the green fluorescent protein (GFP) reporter, SFV vectors allow the quantification of gene delivery **Objectives:** To evaluate by fluorescence microscopy the kinetics of expression of GFP in BHK-21 cells infected with SFV-GFP. **Methods:** The recombinant SFV were produced *in vitro* by transfection of BHK-21 with expression and structural SFV RNAs. After 24 h SFV-GFP were harvested from supernatant and quantified by qRT-PCR. BHK-21 cells cultivated in α-mem medium with 10% SFB were infected with SFV-GFP when reached 80% confluence. Cells were incubated at 37ºC in a chamber containing 5% CO_2 coupled to the fluorescence microscope (IX81 Olympus®). During 24 hours, images were acquired in bright field and fluorescence DIC microscopy. **Results and Discussion:** The first GFP-positive cells were detected four hours post-infection. During the incubation period it was evidenced an increasing number of fluorescent cells until 24 hours post-infection. The infected cells showed fluorescence in different times post infection. This is probably due to different amounts of virus that infected each cell, since SFV-GFP is unable to replicate. In general, the percentage of GFP positive cells in the population was low, but the infected cells showed high fluorescence, which may indicate the presence of a heterogeneous cell population, with cells presenting distinct permissibility of infection.

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6.30 Obtention an evaluation of *Piper* compounds in biological models to schistosomiasis mansoni control

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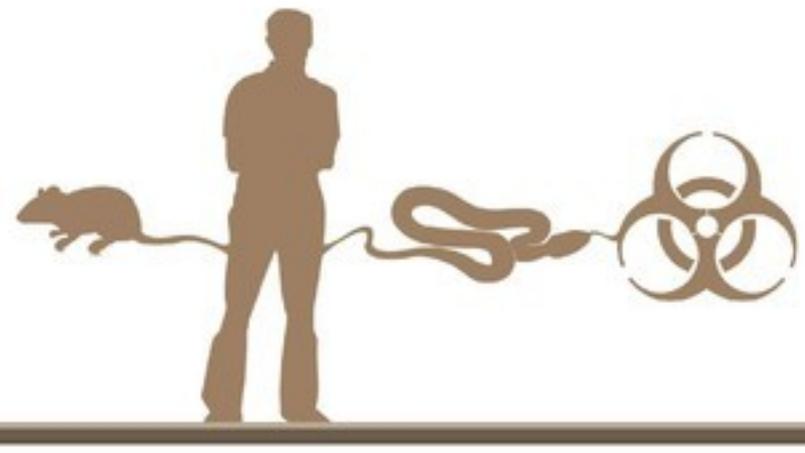
[1]Laboratório de Parasitologia, Instituto Butantan, Brazil; [2]Laboratório de Química e Produtos Naturais, Universidade de São Paulo, Brazil; [3]Instituto de Ciências Biomédicas, Universidade de São Paulo, Brazil

Introduction: Schistosomiasis is an endemic parasitic disease affecting at least 240 million people with more than 779 million at risk of infection. The use of molluscicides has been considered an appropriate method to prevent human infection in hazard areas; however, there is no viable molluscicide avaiable. Studies on Piperaceae bioprospecting identified *Piper* species potentially active in *Biomphalaria glabrata*. **Objectives:** The aim of this study was to search for compounds in *Piper* species active in *B. glabrata* adults and embryos stages and to evaluate schistosomicidal activity in miracidia and cercariae of *S. mansoni* and toxicity of the most active compound in *Daphnia similis* and *Danio rerio*. **Methods:** The active compounds were obtained from *Piper diospyrifolium* bioguided fractionation, which resulted in isolation and identification of two molluscicidal compounds: the flavokavain A, isolated in this species for the first time and the 4-hydroxy-3-[3,7, trimetildodeca-11-2 ,6,10-trienil] benzoic acid, a novel chemical structure. The search for active compounds was carried out in amides, as the most representative group of *Piper* compounds, and chalcones, since the bioguided fractionation of *P. diospyrifolium* has identified flavokavain A as active in *B. glabrata*. **Results and Discussion:** Of the eight compounds evaluated in this study (2 ', 4', 6'-trihydroxydihydrochalcone, dihydroflavokavain C, flavokavain A, 4-hydroxy-3-[3,7,11-trimetildodec-2 ,6,10-trienyl] benzoic acid, pelitorin , piperine, peperlonguminine and piplartine), four were active in concentrations recommended by WHO for molluscicides (2 ', 4', 6'- trihydroxydihydrochalcone, flavokavain A, 4-hydroxy-3-[3,7,11-trimetildodec-2 ,6,10-trienyl] benzoic acid and piplartine. Piplartine amide was the most active in *B. glabrata* adults and embryos, being, therefore the selected compound for evaluation of schistosomicidal activity toxicity. There was no mortality of miracidia and cercariae exposed to piplartine in concentrations below 20 ppm; however changes in the mobility of both organisms at all concentrations were observed. Piplartine was classified as toxic to *D. similis* and *D. rerio*; nevertheless it was less toxic than niclosamide, the only commercially available molluscicide. In this study, molluscicidal compounds were obtained from bioguided fractionation of *P. diospyrifolium* and by evaluating the activity of amides and chalcones from *Piper*. Both methods were suitable to obtain active compounds.

Supported by: FAPESP, CAPES



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6.31 Using the system Semliki Forest Virus for expression of the Rabies virus glycoprotein in mammalian cells

Rezende AG[1], Bernardino TC[1], Astray RM[1], Núñes EGF[1,2], Puglia ALP[1], Pereira CA[1], Lemos MAN[1], Suárez SFP[1], Jorge SAC[1]
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Introduction: The Semliki Forest Virus (SFV), the genus *Alphavirus* is widely used as a vector expression of heterologous proteins in mammalian cells. The rabies virus glycoprotein (RVGP), recognized as an antigen capable of conferring immune response against rabies, was chosen as a target gene in this approach. **Objectives:** To establish protocol for optimization of transfection of viral RNA to produce recombinant SFV (SFV-RVGP); to analyze the RVGP expression in cells infected by SFV-RVGP by using the ELISA; to determine the best conditions for cell culture and viral infection for the expression of the heterologous protein. **Methods:** Two different plasmids were used: an expression plasmid containing SFV genes coding for nonstructural proteins and the RVGP gene, and a helper plasmid containing SFV genes coding for structural proteins. *In vitro* transcription was performed and RNAs were co-transfected in BHK-21 cells, for generation of SFV-RVGP. They were then activated and used to infect BHK-21 and Huh 7.0 cells, and induce the heterologous protein (RVGP). Expression evaluation was done by ELISA. **Results and Discussion:** Using the SFV-RVGP method of expression, we evaluated the time of SFV-RVGP generation, and the RVGP production after infection. The experiments were performed in duplicate in 6 wells plate in CO2 incubator at 37 ° C. The cell inoculum was of 7x1E5 cells/well with a working volume of 2 mL. Based on these results, the method of transfection electroporation was better than the method with a commercial kit Transmessenger, and time 24 hours after transfection, the best collection of viruses generated. Another important fact is that protein production, 48h after infection was higher as compared with that obtained after 24 hours, and that a greater amount of RVGP was generated in a process of infection with BHK-21 cells, when compared to Huh 7.0 cells.

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6.32 Sublethal stimulus induce resistance against cadmium and heat in *Biomphalaria glabrata* (SAY, 1818) snails

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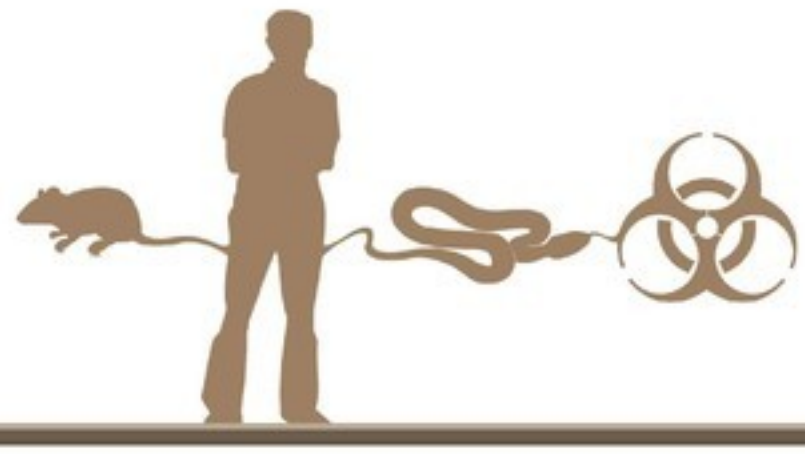
Introduction: Extracellular mild stresses induce heat shock response and can protect cells against subsequent administration of other lethal stresses, rendering cells resistance. This phenomenon is called cross-resistance. Heat shock response involves induction of heat shock proteins (HSPs).**Objectives:** In this research we proved that sublethal expositions to heat and to doses of cadmium chloride improved the survival of snails *Biomphalaria glabrata* to lethal temperature and a lethal concentration of cadmium chloride, respectively; probably activating protection mechanisms involved in the maintenance of life in this organisms. **Methods:** We used pigmented snails, six month old, with shell diameter of 14 (±1,8) mm, exposed to 33 °C and to 0,22 ppm of $CdCl_2$, and challenged with 0,7 ppm of $CdCl_2$ and 42 °C, respectively, as these stimulus were proved lethal to this mollusc. **Results and Discussion:** The results showed an increment in survival of snails pre-exposed to sublethal stimulus, as the control group survived for no more than 96 hours to $CdCl_2$ at concentration of 0,7 ppm and for 5 hours at 42 °C, while in the pre-exposed groups the mean time of survival was higher to both stimulus. This mollusc was chosen because its appliance in many previous experimental protocols in diverse areas, despite the indicatives of it application in environmental investigations. The western blot pointed to an induction of HSP70 protein in groups pre-exposed as compared to the control ones. It is well known the role of HSP70 in thermotolerance and tolerance to other agents of stress in living organisms, and it was proved in *Biomphalaria glabrata* too, adding more subsides to the employment of this snail in environmental monitoring.



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6.33 Development and standardization of immunochromatographic test for Shiga toxin-producing *Escherichia coli* (STEC) and enterotoxigenic *Escherichia coli* (ETEC) diagnosis

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Introduction: Globally, diarrhea is considered the second leading cause of death in children less than five years with 800,000 deaths annually. Among the pathogens, diarrheagenic *Escherichia coli* are responsible for 30 to 40% of diarrhea episodes. Among these, Shiga toxin-producing *Escherichia coli* (STEC) and enterotoxigenic *Escherichia coli* (ETEC) produce potent toxins harmful to man. The diagnosis is an important tool for the correct treatment and outbreaks control. Comparing molecular versus immunoserological methods, such as immunochromatographic assay (IC) it has several advantages, easy implementation, low cost and rapid tests. **Objectives:** Development and standardization of test ICs for detection of ETEC and STEC by their virulence factors: the heat labile toxin (LT) and the Shiga toxin (Stx1 and Stx2), respectively. **Methods:** Rabbit polyclonal antibodies (PAb) against each toxin were previously obtained and characterized in our laboratory. Murine monoclonal (MAb) were characterized by ELISA (IgG isotype and dissociation constant) and the reactivity with their respective antigen was assessed by immunoblotting. The IC test strips were assembled using a sequence of three types of papers: cellulose fiber, glass fiber and nitrocellulose. In the IC test development, the MAbs anti-LT, anti-Stx1 and anti-Stx2 and the corresponding PAbs were conjugated to colloidal gold and the strips of the IC test were assembled and pre-treated. In the standardization of IC test different positions of the MAbs and PAbs were tested, as well as different concentrations of antibodies in the test line. Detection limits with purified toxins and supernatant of bacterial cultures were also assayed. **Results and Discussion:** The positivity of the IC test was obtained after assembly of the strips with the MAb anti-LT (IgG2b, 4.9×10^{-11} M), anti-Stx1 (IgG1, 2.5×10^{-10} M) and anti-Stx2 (IgG1, 6.1×10^{-10} M) in the test line position (capture) and the PAb conjugated to colloidal gold. In these conditions, after 20 min, the test detected 15.6 ng/mL of LT, 15.6 ng/mL of Stx1 and 62.5 ng/ml of Stx2. The three ICs tests showed no cross-reactivity with non-producers isolates of the respective target toxin. The results demonstrate the feasibility and applicability of these tests as diagnostic tools for ETEC and STEC, especially in locations with few laboratory resources.

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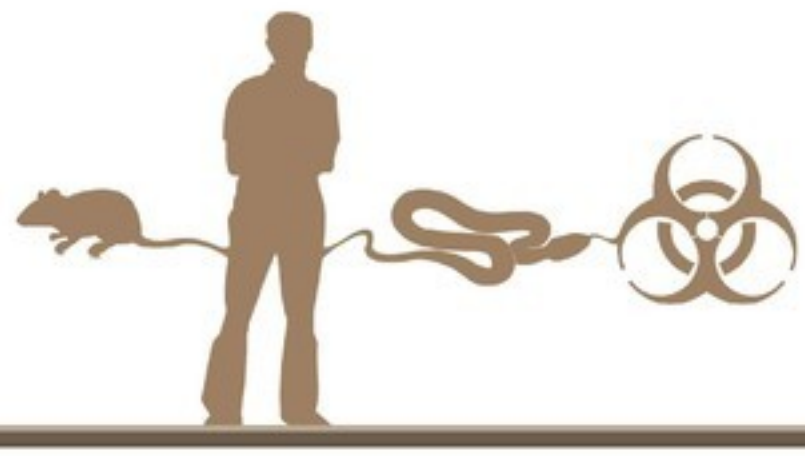

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6.34 Laboratorial scale production of HPV16 L1 protein as a vaccine component against HPV and associated cancers

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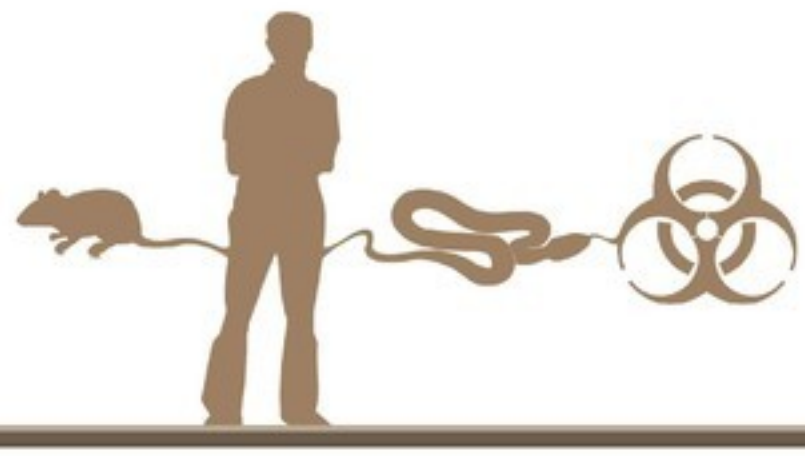
Introduction: Cervical cancer is the most serious consequence of HPV infection. It is the second most common cancer among women and represents an important problem for global public health. HPV16 and HPV18 high-risk types are the most frequently associated with cervical cancer and are responsible for 70% invasive cervical cancers cases. HPV16, the most oncogenic type, is used for the development of prophylactic vaccines against cervical cancer and HPV-associated infections. Currently available vaccines are based on the major capsid protein L1 that self-assembles into virus-like particles (VLP) which can induce neutralizing antibodies. The prophylactic vaccination on a large scale would be an alternative to reduce rates of cervical cancer, making it essential to search for new vaccine strategies. **Objectives:** To produce (VLPs) containing the major capsid protein L1 (VLP L1) HPV16 using cells in suspension for laboratorial scale, as a component of prophylactic and therapeutic vaccine against HPV and associated cancers. **Methods:** In this study, human embryonic kidney cells (HEK 293 F) were cultivated in suspension with serum-free medium based on orbital shaker technology. The 293 F cells were transfected with DNA expression vector encoding for L1 of HPV16. We have analyzed the intracellular protein expression of HPV16 L1 using confocal and transmission electron microscopy. **Results and Discussion**: Preliminary results for L1 protein production in serum free suspension culture are encouraging. We are developing a laboratorial scale process for protein production using suspension cell cultures with orbital shaker technology. The cells in suspension were able to produce high levels of recombinant protein L1, with efficient transfection and expression. The present work provides an interesting alternative to prophylactic and therapeutic vaccine against HPV, with a production of HPV16 L1 on a broader-scale for the benefit of the society and public health.

Supported by: FAPESP, Butantan Institute, Butantan Foundation, CAPES and PAP-SES-FUNDAP Fellowships



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6.35 Immunochromatographic assay for detection of *Streptococcus pneumoniae*

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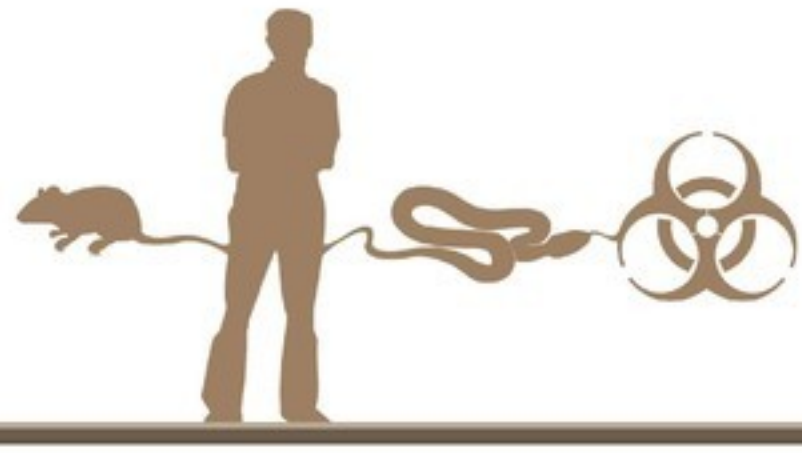
Introduction: *Streptococcus pneumoniae* is a gram-positive diplococcus that causes several invasive diseases, including pneumonia, bloodstream infections and meningitis, accounting for about 4 million deaths per year in developing countries. Traditional techniques for detecting the organism, in spite of its proven utility and reproducibility require an appropriate laboratory, time consuming and specific technical training. Simpler, faster and cheaper alternatives in the diagnosis of pneumococci continue to be of great importance. Immunochromatography strip tests, or namely lateral flow tests, are simple devices, using coloured particles and based on capillary action, that intend to detect, in a few minutes, the presence or absence of target analytes. The most frequently used particle in these assays is the nanometer sized colloid gold. **Objectives:** The aim of this study is the standardization of a rapid immunochromatographic assay (dipstick) using textile colloidal dye as a visualization agent for detection of *S.pneumoniae*. **Methods:** Suspensions of colloidal dye particles (Dianix Brilliant Blue R) were prepared using washing and centrifugation procedures, basically according to reported before by Snowden and Hommel (1991). The optimum wavelength for quantification of the dye particles was determined by spectrophotometric scan. The dye particles were then conjugated, by simple adsorption, to different mice monoclonal antibodies (AcMo) against streptococcal pneumolysin (Ply); a mice polyclonal serum anti-Ply (AcPo anti-Ply) or a mice serum against a whole cell pneumococcal vaccine, in development in our laboratory (anti-WCPV). Multiple buffers systems, with different pH range, were evaluated to determine the best conditions of adsorption. In the dipstick, the colloidal dye particles, conjugated to the antibodies, binds to the antigen sample (Ply or suspensions of *S. pneumoniae*) to form a complex which is captured by the capture antibody (AcMo anti-Ply; AcPo anti-Ply or anti-WCPV) immobilized in a nitrocellulose membrane, under previously determined optimal conditions. Anti-mouse IgG was also immobilized in the nitrocellulose membrane, as control line. **Results and Discussion**: The system was able to detect Ply of *S. pneumoniae* at a concentration of [100µg/ml] and also *S. pneumoniae* as cell suspensions in PBS, but some background was observed in the control negative test. Based on these preliminary results, we expect the completion of the standardization procedures, increasing the sensitivity and specificity of the test, to validate its applications in clinical analysis, as an easy and low-cost technology for the diagnosis of *S. pneumoniae*.

Supported by: CNPq and Fundação Butantan



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6.36 A multifunctional outer membrane protein of *Leptospira interrogans* binds host fibronectin, laminin, plasminogen, fibrinogen and complement regulators

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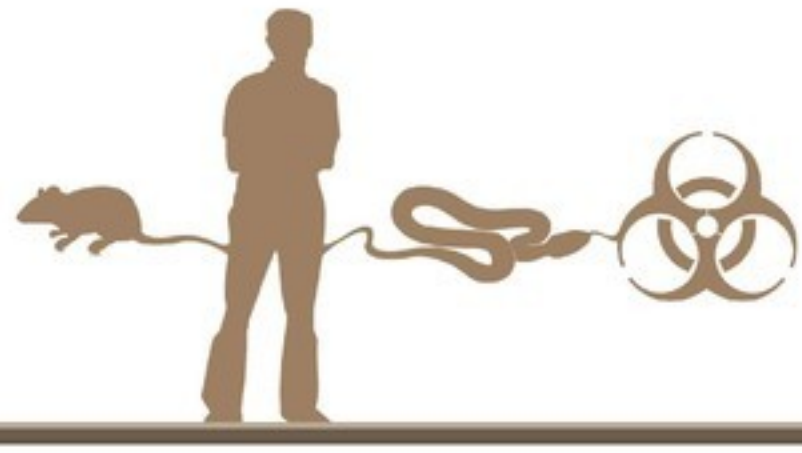
Introduction: Leptospirosis is an emerging infectious disease caused by pathogenic species of the genus *Leptospira*. After invading, the bacteria need to survive from the host's innate immune defense in order to colonize target organs. The adherence of leptospires to components of the extracellular matrix (ECM) may be the principal mechanism for the colonization of host tissues, and surface proteins may play a role in these interactions. **Objectives:** This work aims to clone, express, purify and evaluate the capacity of a recombinant protein (rLIC11360) to attach to ECM macromolecules and serum and plasma components. **Methods:** The gene was cloned and expressed in *Escherichia coli* strain BL21 (SI) using the expression vector pAE. The recombinant protein was expressed tagged with N-terminal hexahistidine, thus facilitating their purification by metal-affinity chromatography. The attachment of purified rLIC11360 to ECM and serum and plasma components was evaluated by binding assays. A screening with immobilized components on 96-well microdilution plates was assessed using ELISA. In order to characterize the binding specificity, dose-response curves were performed by keeping the component and varying the protein concentration. The effect of heparin and lysine analog 6-aminocaproic acid (ACA) on the interaction of recombinant protein with fibronectin and plasminogen (PLG), respectively, was assessed. Plasmin enzymatic assay was performed using uPA (urokinase) -type plasminogen activator and plasmin-specific chromogenic substrate. The inhibitory effect of rLIC11360 on leptospiral binding to ECM, serum and plasma components was performed by ELISA. **Results and Discussion:** The rLIC11360 binds to laminin, plasma fibronectin, PLG, fibrinogen, factor-H and C4bp. These interactions were specific, dose-dependent and saturable. ACA (2 mM) inhibited rLIC11360-plasminogen binding showing the participation of lysine residues in this interaction, and the plasminogen captured by rLIC11360 could be converted into plasmin. Live leptospires binding with laminin, fibronectin, PLG, fibrinogen, and complement regulators were inhibited by different concentrations of rLIC11360. This protein was previously genome annotated as putative outer membrane proteins of *L. interrogans* of unknown function. Our results show that this novel protein is an adhesin that may play a multifunctional role in pathogenesis of leptospirosis, helping the bacteria to adhere and to spread through the hosts.

Supported by: FAPESP, CNPq and Fundação Butantan



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6.37 Adaptation of mammalian cells in serum-free media

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Introduction: The use of fetal bovine serum (FBS) as supplement for cell culture often is still necessary for optimal cell growth and subsequent protein expression. However, the serum is a highly complex compound, hence it has some drawbacks such as increased cost of the culture medium, variability from lot to batches, introduction of contaminants and difficulties in recovery and purification process of the bioproduct. For these reasons, new serum-free media have been developed and tested in different animal cells under previous adaptation. **Objectives:** To study the kinetic behavior of mammalian cell lines adapted to serum-free media (SFM) for the optimization of recombinant proteins expression, rabies virus glycoprotein (RVGP) and the non structural protein of hepatitis C virus (NS3), through a gene expression system derived from Semliki Forest virus. **Methods:** BHK-21 cells switched from serum-supplemented medium (DMEM with 10%FBS) into different commercial SFM (VP-SFM, Hybridoma-SFM and CHO-S-SFM II) in several steps. **Results and Discussion:** The adaptation to SFM was performed gradually over several subcultures to avoid harming the cells. Decreasing the serum concentration gradually were obtained cells adapted to growth in VP, Hybridoma and CHO Serum-free media supplemented with 1% of FBS. Kinetics parameters of these cells were analyzed in duplicate for five days in 6 well plates with 2 mL of medium, the initial cell inoculum was 2×10^5 cells/mL. The maximum cell concentrations reached after 96 hours of culture were 2.6×10^6 cells/mL; 2.3×10^6 cells/mL; 2.1×10^6 cells/mL and 2.5×10^6 cells/mL for Hybridoma, CHO-SFM II, VP-SFM and Control (DMEM with 10% of FBS) respectively. The concentrations achieved in all three SFM were similar to those obtained in the control with 10% FBS, suggesting a good adaptation to growth of BHK-21 cells in media studied.

Supported by: FAPESP

6.38 Cloning and expression of a new OmpA-like protein of *Leptospira interrogans*

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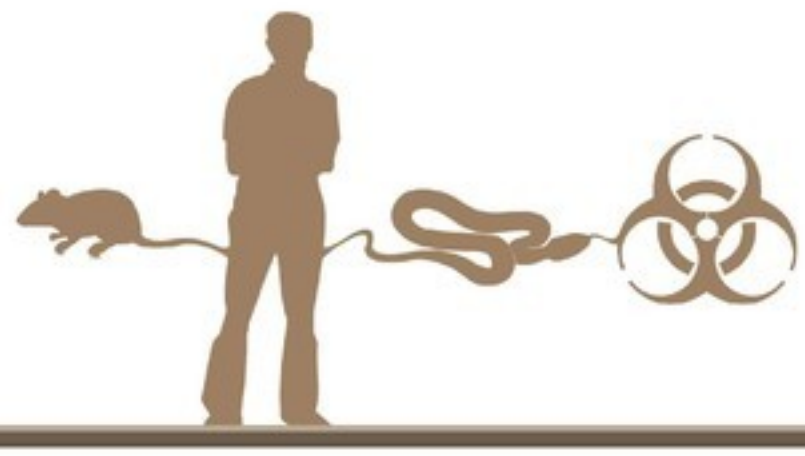
Introduction: Leptospirosis is a worldwide zoonosis regarded as a major public health problem. In Brazil, outbreaks occur mainly in rainfall periods in metropolitan areas that have inadequate infrastructure favoring the proliferation of rodents which are the main reservoirs of the disease. Measures to control the disease are difficult to implement. The development of new strategies to prevent and control the spread of leptospirosis is urgently needed. Accordingly, prophylactic vaccines or immunotherapeutic emerge as a strong candidate to solve the problem. Only Cuba and China have licensed vaccines for humans, but these formulations are based on inactivated leptospires presenting a series of side effects and are serovar-dependent. For this reason, currently research has focused to identify conserved antigens that are involved in host-pathogen interactions. **Objectives**: This project aims to assess the functional properties of the gene (LIC13479) identified in the genome sequences of *Leptospira interrogans* serovar Copenhageni. **Methods:** The LIC13479 sequence was amplified by PCR using specific primers, cloned into the expression vector pAE and used to transform *E.coli* DH5-α. Ampicilin resistant recombinant clones were selected for plasmid DNA isolation and confirmation by restriction analysis. Plasmids containing the DNA inserts cloned were introduced in *E. coli* strains (BL21SI; BL21 Star (DE3) pLysS; and C43 (DE3)) for protein expression studies. Different concentrations of NaCl and IPTG were tested for induction at 30 ºC and 37°C. **Results and Discussion:** The coding sequence LIC13479 was successfully cloned into *E. coli* expression vector pAE, without the sequences corresponding to the signal peptide. The expected recombinant protein of 46 kDa was detected by 12% SDS-PAGE and was expressed in the insoluble form in all strains used. However, the best expression was obtained in strain *E. coli* BL21 Star (DE3) and BL21 Star (DE3) pLysS with 1mM IPTG at 37°C for 3 hours. Protein purification was successful and produced 1.5mg/L of recombinant protein per bacterial culture. Preliminary studies have shown that the recombinant protein LIC13479 is immunogenic, capable to stimulate antibody immune response in Balb/C mice. Protein secondary structure and stability will be further evaluated in order to characterize the recombinant protein.

Supported by: FAPESP, CNPq and Fundação Buntantan



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6.39 Clinical evaluation and evolution of healing process in mice wound skin treated with *Phyllocaulis boraceiensis* mucus

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Introduction: *Phyllocaulis boraceiensis* mucus has been studied as a potential source of new natural compounds capable of inducing proliferation and remodelling tissue. Human fibroblasts treated with 0.012 µg/µl of *P. boraceiensis* mucus have high rates of proliferation inducing a significant increase in production and secretion of extracellular matrix such as collagen fibres. **Objectives:** The aim was evaluate evolution and clinical aspects of healing wound skin of mice treated with mucus released by *Phyllocaulis boraceiensis*. **Methods:** Briefly, mice were anaesthetized intra mussel by ketamine-xilasine and a 1cm^2 dorsal incision was made under sterile conditions. Mice were treated with papain as control (T_1), papain associated with 0.18 µg/µl of mucus (T_2) and papain associated with 0.012µg/µl of mucus (T_3). During 16 days both groups received a portion of each topical treatment and everyday images were captured of each wound using a dermatoscopy. Measurement of clinical aspects and scar evolution follow a score applied to determine if an aspect was present in high, moderate or low intensity. **Results and Discussion**: Proliferation was accelerated to T_3 being observed after three days presenting high deposition of fibroblasts at wound margin while in T_1 was started five days after surgery. T_2 presented inflammation during all period of observation even when healing had already begun, the new tissue showed capillary fragility. Remodeling was started after four days in T_3 while the others after six days. T_3 showed edema, hyperemia and bleeding only until the 5th day and granulation tissue and scar appear intensely from the 11st day. T_1 and T_2 showed edema, hyperemia and bleeding until the 11st day and granulation tissue and scar appear after 13th day. Results require histological studies however it is possible to infer that dermatological treatment using prescription made up with mucus of *P. boraceiensis* is recommended since this compound proved to be a potential inducer of regeneration.

Supported by: CNPq