5. Microorganisms

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5.01 Validation of sterility test in isolator of *Streptococcus pneumoniae* suspension formulated at Butantan Institute by means of bacteriostatic and fungistatic effect

Agostini Utescher CL, Orozco SFB, Cunha AA, Duboc MA
Serviço de Controle de Qualidade, Instituto Butantan, SP, Brasil

Introduction: Sterility of *Streptococcus pneumoniae* suspension formulated at Butantan Institute for pneumococcal vaccine development is determined by direct inoculation in fluid thioglycollate medium (FTM) and soybean-casein digest media (SCM). The current standard requires that all operational procedures used in quality control must be validated according Current Good Laboratory Practice (cGLP). This procedure ensures analysis and final product quality. **Objectives:** The aim of this study was to determine the sensitivity of bacterial and fungal sterility test (by direct inoculation methodology) applied to *S. pneumoniae* suspension formulated at Butantan Institute, in order to validate the technique used in isolators under Microbiological Control Section conditions. **Methods:** This study was performed using three batches of *S. pneumoniae* suspension formulated at Butantan Institute used in pneumococcal vaccine production. These lots were tested according to standard methodology. After direct inoculation of *S. pneumoniae* suspension, a viable inoculum of ATCC microorganisms (10-100 cfu/mL) was used to validate the technique. FTM was challenged with *Clostridium sporogenes, Pseudomonas aeruginosa* and *Staphylococcus aureus,* and SCM was challenged with *Aspergillus brasiliensis, Bacillus subtilis* and *Candida albicans*. Culture media were incubated for not more than 5 days at 20°C - 25°C and 30°C – 35°C (SCM and FTM, respectively). **Results and Discussion:** Characteristic growth of all microorganisms was determined during an observation period of 5 days. The methodology applied in isolator to bacterial and fungal sterility test of *Streptococcus pneumoniae* suspension formulated at Butantan Institute is effective and demonstrates a high degree of sensitivity to detect low levels of contaminants. We conclude that the methodology can be performed without modification. The formulated product is absolutely safe (sterile) according national and international standards and may be used to vaccinate the public.

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5.02 The influence of kefir on proliferation of protozoa

Ruela AC[1], Anjos MT[1], Seleghim MHR[2], Ruiz RC[1], Melo KCM[1], Domingos MO[1]
[1]Laboratório de Bacteriologia, Instituto Butantan, SP, Brasil; [2]Laboratório de Microrganismos Aquáticos, Universidade Federal de São Carlos, SP, Brasil

Introduction: Kefir is a beverage derived from the fermentation of milk by a symbiotic association of bacteria and yeast which has the ability to inhibit the proliferation of *Candida albicans* and pathogenic Gram-positive and Gram-negative bacteria. However the influence of kefir on protozoa has never been investigated. **Objectives:** The objective of this work was to determine the influence of kefir on the proliferation and morphology of free-living (*P. caudatum*) and pathogenic (*Giardia lambia*) protozoa. **Methods:** The influence of kefir on proliferation of *P.caudatum* was determined by incubating the protozoa at a concentration of 5 organisms/ml in mineral water with 20 µl/ml of milk in the presence or absence of kefir diluted 1:2, 1:4 and 1:8. After 36 and 96 h of incubation at 30°C, the number of protozoa was determined in a Segewich-Rafter chamber, and their morphology was observed by light microscopy. *Giardia lambia* (10^4/ml) were incubated in YI-S Base medium at 30°C for 24, 48, 72 and 96 h. After incubation, the protozoa were fixed with a saturated solution of $HgCl_2$, stained with bromophenol-blue (0.04%) and counted in a Neubauer chamber. Their morphology was also observed by light microscopy. **Results and Discussion:** The results obtained in this work showed that the 1:2 dilution of kefir killed both protozoa. Morphological visualization by light microscopy showed that at this dilution, kefir disrupted the membrane of *P. caudataum* but not the membrane of *Giardia lambia*. Kefir diluted 1:4 inhibited the proliferation of both *P. caudatum* and *Giargia lambia*, but a 1:8 dilution of kefir had no effect on either the proliferation or morphology of these protozoa. In summary, the results obtained in this work suggest that kefir has the potential to be used as a natural remedy against pathogenic protozoa.

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5.03 Preliminary screening for *Leptospira* adhesins through *in vivo* phage display

Ching ATC[1,2], Fávaro RD[1], Lima SS[1,3], Ho PL[1,2,3]
[1]Centro de Biotecnologia, Instituto Butantan, SP, Brasil; [2]Programa de Pós-Graduação Interunidades em Biotecnologia, Universidade de São Paulo, SP, Brasil; [3]Departamento de Bioquímica, Instituto de Química, Universidade de São Paulo, SP, Brasil

Introduction: Leptospirosis is a zoonosis of global importance caused by a pathogenic spirochete. In Brazil, 80% of diagnosed cases are due to *Leptospira interrogans* serovar Copenhageni. Bacterial adhesion to the host is a key event in the infection process. **Objectives:** In attempt to identify the genes coding for adhesins that would bind to some key components of the host extracellular matrix (ECM), we constructed libraries from randomly fragmented bacterial DNA from *L. interrogans* serovar Copenhageni by shotgun for phage display. **Methods:** These libraries were prepared into a phagemid vector and screened in vivo using Golden hamsters. The hamsters were anesthetized, and 200 µL of phage were injected through the left ventricle of the heart and allowed to circulate for 5 min. Animals were perfused with citrate buffer until the perfusate was clear and the organs appeared free of blood. Organs and tissues were placed in DMEM supplemented with protease inhibitors, weighed, homogenized and amplified by infection into bacteria. The phage was titered and the same input phage dose was used for each round of panning. **Results and Discussion:** The phage was titered and the same input phage dose was used for each round of panning. About 100 clones from each of the 4 rounds were sequenced. Candidates with extracellular binding properties were chosen and tested by in vitro binding assays with ECM molecules. This is the first report about screening *L. interrogans* adhesins using in vivo phage display.

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5.04 Application of BacT/ALERT 3D in production process control of diphtheria, tetanus and pertussis vaccine (DTP)

Agostini Utescher CL[1], Orozco SFB[1], Duboc MA[1], Chaimovick H[2]
[1]Serviço de Controle de Qualidade, Instituto Butantan, SP, Brasil; [2]Fundação Butantan, SP, Brasil

Introduction: BacT/ALERT 3D (B/A) system is an automatic instrument used for early detection of bacterial and fungal contamination, and the flasks containing culture media provides nutritional and environmental conditions suitable for microorganisms growth. This system uses a colorimetric sensor and reflected light to monitor the presence and production of carbon dioxide dissolved in the culture medium, produced as a result of microbial metabolism. The DTP (diphtheria/tetanus/pertussis) vaccine is part of the national immunization schedule used for active immunization of children. Traditional evaluation of bacterial and fungal sterility of this product is carried out by membrane filtration (FM) technique and visual analysis over 14 days. **Objectives:** The aim of this study was to apply the B/A system during the DTP vaccine production process, aiming to reduce retention time and to demonstrate the importance of preservative neutralization (thimerosal) by means of bacteriostatic and fungistatic effect on detection sensitivity of low levels of microorganism contaminants. **Methods:** We used three batches of DTP production previously approved on bacterial and fungal sterility test by traditional methodology. We used BacT/ALERT SN and SA culture media, with a sample volume of 1.0 mL plus 1.0 mL of DNP (Diluent Neutralizing Pharmacopoeic fluid), injected directly into the culture medium and kept under observation for 14 days. Each lot was challenged with a 10-100 CFU/mL inoculum of the following microbial strains: *Aspergillus brasiliensis, Bacillus subtilis, Candida albicans, Clostridium sporogenes, Pseudomonas aeruginosa* and *Staphylococcus aureus.* The sensitivity of the culture media was tested with negative control (DNP sterile solution) and positive (ATCC microorganism suspension without the product being tested). **Results and Discussion:** When neutralized by DNP solution, the three lots tested revealed the following recovery times: *A. brasiliensis* 165.4 h; *B. subtilis* 18.9 h; *C. albicans* 72.4 h; *P. aeruginosa* 21.4 h; *S. aureus* 31.4 h and *C. sporogenes* 47.0 h. The negative control showed no microbial growth and the positive controls (pure cultures), recovery times as follow: *A. brasiliensis* 30.0 h; *B. subtilis* 14.6 h; *C. albicans* 17.8 h; *P. aeruginosa* 14.4 h; and *S. aureus* 32.0 h. The results show that the B/A system can only be used in production process control for DTP vaccine when added with a neutralizing preservative solution. We observed that the recovery time of fungal growth in SA medium and *C. sporogenes* in SN medium is greater than in positive controls. The results show that B/A system can be used to control the production process, significantly reducing product retention time. Inhibitory components of microbial growth incorporated on product composition are completely neutralized by DNP and B/A system, allowing low levels of contamination detection.

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5.05 Cloning and analysis of genes that encode proteins identified by mass spectrometry of atypical enteropathogenic *Escherichia coli* of serotype O55:H7

Souza CS, Ogawa R, Piazza RMF
Laboratório de Bacteriologia, Instituto Butantan, SP, Brasil

Introduction: Enteropathogenic *Escherichia coli* (EPEC), one of the six *E. coli* diarrheagenic (DEC) pathotypes produces an adherence factor chromosomally encoded by the *eae* (EPEC attaching and effacing) gene located within the locus for enterocyte effacement (LEE) pathogenicity island and have been categorized into two subgroups termed typical and atypical EPEC. Typical EPEC (tEPEC) strains contain, in addition to *eae*, the EPEC adherence factor (EAF) plasmid, which encodes the bundle-forming pili that mediate localized adherence to epithelial cells. EPEC strains lacking the EAF plasmid and non-expressing BFP have been designated atypical EPEC (aEPEC). **Objectives:** Previous data generated by proteomics analysis, using the technique of peptide mass fingerprint, showed differences between outer membrane proteins (OMPs) from aEPEC serotype O55:H7 and tEPEC serotype O55:H6, Thus, in the present study, four proteins expressed only in aEPEC (spots 114, 135, 192 and 271) were chosen for gene cloning in expression systems and protein characterization. **Methods**: The primers to amplify these genes were designed with the Gene Runner program, and PCR was performed using Platinum Pfx Polymerase (Invitrogen) using standard amplification conditions. The amplified products were purified and cloned into cloning vector pGEM-T Easy vector. These plasmids containing the inserts were transformed into *E. coli* JM109. The confirmation of cloning was performed by restriction analysis with the enzymes HindIII and NdeI. **Results and Discussion:** Among the target proteins, three have identity with proteins of unknown function and one is a mobilization protein. The protein sequences were obtained through its gene identification (gi=in http://blast.ncbi.nlm.nih.gov) and were analyzed by the presence of signal peptide and transmembrane regions using the programs Signal P and DAS transmembrane, respectively. The analysis showed that these sequences have no signal peptide and only the hypothetical Fpla064 protein (spot 114) showed five transmembrane segments. The presence of conserved domains was confirmed with the program Conserved Domains. Thus, the protein 114 showed conserved domains of super family ParBC and SpoOJ; the protein 192 showed conserved domains of mobilization proteins. Oligonucleotides were designed using the gene sequences of each protein, and these oligonucleotides were used for amplification of the respective genes, where only three genes were amplified. One of the generated fragments (spot 135) was cloned in cloning vector pGEM-T Easy vector for later subcloning in expression vectors. The characterization of these proteins should contribute to understanding the virulence mechanism of aEPEC of serotype O55:H7, since these target proteins were identified only in aEPEC and were absent in serotype O55:H6.

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5.06 Construction and analysis of atypical enteropathogenic *Escherichia coli* QseC mutants

Franzin FM[1,2], Brocchi M[2], Sircili MP[1]
[1]Laboratório de Bacteriologia, Instituto Butantan, SP, Brasil; [2]Programa de Pós-Graduação em Genética e Biologia Molecular, Universidade Estadual de Campinas, SP, Brasil

Introduction: Quorum sensing (QS) is a cell signaling mechanism in which bacteria produce and detect extracellular signaling molecules called autoinducers (AI). This mechanism is used by gram-positive and gram-negative bacteria to regulate many of physiological functions. In pathogenic bacteria, QS controls the gene expression of virulence factors. Many QS systems have been identified in different bacterial species. One of these, described in enterohemorrhagic *Escherichia coli* (EHEC), involves the detection of AI-3 and the mammalian hormones epinephrine and norepinephrine. The pathogens use the sensor histidine kinase, QseC (quorum sensing *E. coli* C) to recognize both host-derived signals and the bacterial signal to activate their virulence genes. Upon sensing these molecules, QseC autophosphorylates and phosphorylates the transcription factor QseB, which initiates a regulatory cascade and leads to the transcription of genes such as flagella, adhesins, and the genes of the pathogenic island LEE (locus of enterocyte effacement). The LEE region contains most of the genes involved in the attaching and effacing (AE) lesion, the intestinal histopathology associated with enteropathogenic *E. coli* (EPEC) and EHEC infections. **Objectives:** The purpose of this study was to construct strains of atypical EPEC with a mutation in the *qseC* gene for further studies of its influence on gene regulation of virulence factors encoded on LEE. **Methods:** The *qseC* mutagenesis in two atypical EPEC strains was obtained using homolog recombination technique based on bacteriophage λ Red system. In this technique, the *qseC* gene was replaced by the recombination cassette that contains the *cat* (chloramphenicol acetyl transferase) gene generated by PCR amplification. This allelic exchange was mediated by enzymes that inhibit the degradation and promote recombination of linear DNA target. To confirm the recombination, two PCR amplifications were performed using specific primers: to the *cat* genes and to the flanking region of *qseC* gene. **Results and Discussion:** The recombination generated in mutant strains an amplification of *cat* gene (900 bp), while in the wild type strains, no amplification was detected. When the primers external to the *cat* gene were used in the amplification, the mutant strains showed an amplicon of 1,100 bp, corresponding to the *cat* gene, while the wild type strains showed an amplicon of 1,300 bp corresponding to *qseC* gene. Using adherence assays with cultured epithelial cells, we showed that QseC mutants are capable of forming AE lesions, but not as efficient as wild type strains. Further studies will help us to elucidate the role of QseC in the AE lesion.

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5.07 Development of transcriptional fusions to assess *Leptospira interrogans* promoter activity

Cerqueira GM[1], Souza NM[1], Araújo ER[1], Barros AT[1], Morais ZM[2], Vasconcellos AS[2], Picardeau M[3], Nascimento ALTO[1,4]
[1]Centro de Biotecnologia, Instituto Butantan, SP, Brasil; [2]Laboratório de Zoonoses Bacterianas do Departamento de Medicina Veterinária Preventiva e Saúde Animal, Faculdade de Medicina Veterinária e Zootecnia, [4]Programa de Pós-Graduação Interunidades em Biotecnologia, Universidade de São Paulo, SP, Brasil; [3]Unité de Biologie des Spirochètes, Institut Pasteur, France

Introduction: Leptospirosis is a zoonotic infectious disease that affects both humans and animals, and has emerged as a major public health problem in developing countries. More than 500,000 cases of severe leptospirosis are reported each year, for which the mortality rate is up to 50%. The genome sequences of pathogenic and saprophytic *Leptospira* revealed the existence of several predicted coding sequences of unknown function. The study of *Leptospira* pathogenesis has been hampered by the lack of appropriate genetic tools. Recent advances have been made regarding the genetic manipulation of *Leptospira*, improving our understanding of the biology of this spirochete, but the need to develop basic molecular systems for the assessment of *Leptospira*–host interactions still remains. **Objectives:** The aim of this study was to develop a novel genetic tool to study leptospiral promoter activity under mammalian host conditions. **Methods:** A series of replicative promoter-probe vectors carrying a reporter gene encoding green fluorescent protein (GFP) fused to the leptospiral promoters of the genes *lipL41*, *ligA* and *sph2* were constructed for use in *L. biflexa*. The *L. biflexa* reporter strains were induced by physiological osmolarity and temperature, urine pH 6.7 and the supplementation with spermine (either individually or in combination). Promoter activity was assessed by fluorescence measurement, RT-PCR and epifluorescence microscopy. The validation of the conditions tested was performed by inducing LigA expression in the pathogenic *L. interrogans* serovar Copenhageni strain L1-130. Native protein expression was assessed by ELISA and RT-PCR. **Results and Discussion:** The *ligA* and *sph2* promoters were the most active promoters, in comparison to the *lipL41* promoter and the non-induced controls. The results obtained are in agreement with LigA expression from *L. interrogans* Fiocruz L1-130 strain, which was cultivated under the same defined growth conditions. The constructed vectors facilitated the *in vitro* measurement of *L. interrogans* promoter activity under defined growth conditions which simulate the mammalian host environment. The fluorescence and RT-PCR data obtained closely reflected transcriptional regulation of the promoters, thus demonstrating the suitability of these vectors for assessing promoter activity in *L. biflexa*.

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5.08 Analysis of biofilm formation by atypical enteropathogenic *Escherichia coli* strains by CFU count, crystal violet assay and confocal fluorescence microscopy

Culler HF, Mota CM, Abe CM, Sircili MP, Franzolin MR
Laboratório de Bacteriologia, Instituto Butantan, SP, Brasil

Introduction: Microorganisms can proliferate as individual cells swimming freely in the environment, or they possess the capacity to adhere, forming biofilms in association with different types of surfaces and interfaces and conferring resistance to some antibiotics. Many models have been described for the study of development of biofilm *in vitro*. The most common procedure used to measure the biofilm formation is counting viable cells on plates; however, several tests for biofilm quantification in microtiter plates, as the method of crystal violet (CV) staining, have been recently described. Another technique often used is confocal laser scanning microscopy (CSLM), as a qualitative method. **Objectives:** The aim of this study was to determine the capacity of biofilm formation by atypical enteropathogenic *E. coli* (EPEC) on an abiotic surface by CFU/cm^2 counting, colorimetric assay of CV and CSLM with fluorescent marker after 24 h of incubation at 37°C. **Methods:** A total of 12 atypical EPEC strains isolated from children with diarrhea were studied after growing in high glucose DMEM. The biofilm formation of the bacterial strains was studied in static conditions by the crystal violet assay (CV) using polystyrene 24-well culture plates and was quantified at 595 nm in an ELISA plate reader. These strains also were tested by CFU/cm^2 counting attached to the biofilm after disrupting with Triton X-100. Serial dilutions were made and plated on Luria-Bertani agar for CFU/cm^2 counting. The strains were also visualized by CSLM, after the staining the bacteria red with propidium iodide. **Results and Discussion:** Six of the 12 strains showed optical density (OD) values above 0.100, and 6 showed OD values below 0.100. Through the analysis of fluorescence confocal microscopy, it was possible to visualize strains that were capable of forming biofilms and others that did not, as well as this biofilm structure. The CLSM and CV methods showed similar results. The bacterial strains showed constant CFU/cm^2 values in the range of 10^8, and compared with the analysis of images obtained with CSLM, there was no agreement between the two methods. The CV assay measured live and dead cells, while these cells may be distinguished by CLSM and CFU/cm^2 counts measure only the viable cell number. The CFU/cm^2 count and CV methods should not be used alone to indicate biofilm formation, but should be used together with a qualitative method such as CLSM, which allows the visualization of biofilm structures and measures biofilm thickness.

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5.09 Expression of TNF-alpha and CXCL-2 mRNAs in different organs of mice infected with pathogenic *Leptospira*

Da Silva JB[1], Carvalho E[1], Ching ATC[1], Moreira VB[2], Mattaraia VGM[2], Pereira MM[3], De Franco M[4], Favaro RD[1], Vasconcellos S[5], Ho PL[1], Martins EAL[1]
[1]Centro de Biotecnologia, [2]Biotério Central, [4]Laboratório de Imunogenética, Instituto Butantan, SP, Brasil; [3]Fundação Instituto Oswaldo Cruz, RJ, Brasil; [5]Faculdade de Medicina Veterinária e Zootecnia , Universidade de São Paulo, SP, Brasil

Introduction: Leptospirosis is a worldwide zoonosis caused by *Leptospira*. The infection in humans is mainly observed in lungs, kidneys and livers. Studies of immune response to leptospira infection can provide information for proper vaccine development and elucidation of the mechanisms of pathogenesis and can contribute to the development of more effective treatment and prevention of the disease. Injury by microbial factors and the production cytokines and chemokines in response to infection have been proposed to be involved in the pathogenesis of leptospirosis. Among the chemokines and cytokines, CXCL-2 (also called macrophage inflammatory protein 2 - MIP-2) and TNF-α are produced in inflamed tissues and they coordinate the migration and accumulation of leukocytes at the inflammatory sites. **Objectives:** In the present study, gene expression of cytokine TNF-α and chemokine CXCL-2 in kidney, liver and lung of mice infected with *Leptospira* was investigated. **Methods:** Mouse strains C3H/HeJ, C3H/HePas and BALB/c were infected i.p. with $2{,}4x10^6$ virulent *Leptospira interrogans* serovar Copenhageni. Three mice of each strain (n=12) were sacrificed on days 0 (uninfected), 1, 3, 5 and 7 after infection. Total RNA was isolated from the tissues and the relative expression of TNF-α and MIP-2 and GAPDH was analyzed by real-time quantitative PCR. Relative expression of the genes was calculated by the $2^{-\Delta\Delta}$Ct method using GAPDH mRNA for normalization. **Results and Discussion:** Our results indicate that the expression of MIP-2 and TNF-α can vary greatly, depending on the tissue, mouse strain, and time of infection. For instance, the expression of TNF-α and MIP-2 rapidly increased on day 3 (40- and 60-fold, respectively) in the liver of BALB/c, returning to baseline levels on day 5. This response can be associated with the resistance phenotype of this mouse strain to leptospirosis.

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5.10 Atypical enteropathogenic *Escherichia coli* strains secrete an inhibitor of phagocytosis

Melo KCM[1], Marques-Porto R[2], Pimenta DC[2], Ruiz RC[1]
[1]Laboratório de Bacteriologia, [2]Laboratório de Bioquímica e Biofísica, Instituto Butantan, SP, Brasil

Introduction: Phagocytosis is important in the establishment of the innate immune defense. Its inhibition is a common escape strategy employed by many pathogens. Typical enteropathogenic *Escherichia coli* (tEPEC) inhibits its own phagocytosis through effector proteins EspF and EspB, translocated into host cells. We have shown that atypical enteropathogenic *E. coli* (aEPEC) O55:H7 is less phagocytized by both lineage and primary culture murine macrophages. **Objectives:** The aim of this study was to investigate the behavior of aEPEC strains 7 and 320 (O55:H7) in J774A1 macrophages. **Methods:** aEPEC and commensal *E. coli* C600 were cultured either in tryptic soy broth or in minimum medium. Macrophages previously incubated with the supernatant from these cultures were infected with tEPEC (E234867). The supernatant from the bacterial cultures was fractionated by solid phase extraction (SPE) in C18 cartridges with 0, 25, 50, 75 and 90% acetonitrile (ACN) containing 0.1% trifluoroacetic acid (TFA). J774A1 were pre-incubated for 30 min with each lyophilized SPE fraction resuspended in RPMI. Active fractions were analyzed and re-fractionated by reversed-phase HPLC in a C8 column and eluted with a 0 to 90% gradient of ACN or methanol in 0.1% TFA. Alternatively, the bacterial culture supernatants were dialyzed against water using membranes with a 1,000 Da cutoff prior to SPE. Fractions from the HPLC were tested for their capacity to interfere in bacterial and *Saccharomyces cerevisiae* phagocytosis as well as in adhesion of tEPEC to HEp-2 and Caco-2 cells. **Results and Discussion:** Only fractions with molecules smaller than 1,000 Da inhibited phagocytosis. These were then analyzed by HPLC and the sub-fractions were again tested for anti-phagocytic activity. Active fractions also reduced phagocytosis of *S. cerevisiae* and adhesion of intestinal extracellular bacteria to epithelial cells, which is an essential step in the establishment of infection. All HPLC fractions eluted with methanol were inactive. The soluble anti-phagocytic factor secreted by aEPEC O55:H7 is relatively hydrophilic and is smaller than 1,000 Da. It is probably not of peptide nature, since it is inactivated when eluted with methanol in acidic conditions, which is known to promote methylation of glucoside groups. It is the first time that an anti-phagocytic mechanism is described in aEPEC. Furthermore, this mechanism differs from that previously described in that it depends on a secreted factor which is soluble in an aqueous medium. This finding opens the possibility of identifying a factor that prevents bacterial adhesion to professional and non-professional phagocytes, which could provide a means of controlling bacterial colonization and preventing diarrhea.

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5.11 Preliminary identification of secreted proteins by two species of *Leptospira*, one pathogenic and one saprophytic

Ricardi LMP[1], Sonobe MH[1], Portaro FCV[2], Morais ZM[3], Vasconcellos SA[3], Barbosa AS[1], Abreu PAE[1]
[1]Laboratório de Bacteriologia, [2]Laboratório de Imunoquímica, Instituto Butantan, SP, Brasil; [3]Laboratório de Zoonoses Bacterianas, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, SP, Brasil

Introduction: Leptospirosis is a zoonosis of worldwide distribution caused by pathogenic spirochetes of the genus *Leptospira*. The mechanisms by which leptospires invade the host and cause the disease are not fully understood, but experimental results have shown that pathogenesis may be related to the ability of these bacteria to bind to extracellular matrix proteins, to escape host's immune responses and to produce toxins. Proteomic analyses of *L. interrogans* have confirmed the theoretical prediction of many proteins possibly involved in the host-pathogen interactions. Up to now, there are no published data of proteins secreted by leptospires identified using proteomic approaches, despite the existence of homolog genes in *L. interrogans* genome that encode for some transport systems known in other bacteria. **Objectives:** This study aimed to identify secreted proteins of *Leptospira interrogans* serovar Pomona strain Fromm (pathogenic) and *L. biflexa* serovar Patoc strain Patoc I (non-pathogenic) by proteomic analyses. **Methods:** *L. interrogans* serovar Pomona strain Fromm, whose virulence was maintained by passages in hamsters, and *L. biflexa* serovar Patoc strain Patoc I were cultured in EMJH medium at 29°C or 37°C. Secreted proteins were collected by centrifugation for removal of cells and subjected to lyophilization. Protein samples were first resolved by IEF on pH 3 to 10, immobilized pH gradient strips (13 cm). Strips were then processed for the second-dimension separation on 12.5% SDS-polyacrylamide gels. Gels were stained with Coomassie blue R-350 or silver. Spots were detected and analyzed by scanning on Labscan (GE Healthcare). *In silico* analyses were performed using SOSUI-GramN and VirGel V.2.0 programs. **Results and Discussion:** Genome-based signal peptide algorithms predicted 179/251 secreted proteins for *L.interrogans* serovar Copenhageni and 161/326 secreted proteins for *L. biflexa* serovar Patoc I with SOSUI-GramN and VirGel V.2.0 programs, respectively. Our 2D-PAGE analyses successfully detected 67 protein spots from the supernatants of serovar Fromm and 211 protein spots from serovar Patoc 1. Further mass spectrometry analyses of the detected spots will be performed. The identification of these proteins will certainly contribute to the elucidation of the pathogenic mechanisms and development of novel strategies for the treatment and prevention of leptospirosis.

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5.12 Biologically active molecules from the hemolymph and fat body of *Lonomia obliqua*

Martins LM[1,2], Moraes RHP[1], Silva Jr PI[2], Mendonça RZ[1]
[1]Laboratório de Parasitologia e Entomologia, [2]Laboratório Especial de Toxinologia Aplicada/CAT-cepid, Instituto Butantan, SP, Brasil

Introduction: Several studies have been conducted demonstrating the presence of pharmacologically active substances in the hemolymph with enzymatic, hormonal, antimicrobial and anti-freeze effects. The fat body is the main organ of intermediary metabolism of insects and the main source of components of the hemolymph. It facilitates the process of detoxification, and is involved in the production and secretion of lipids in the hemolymph, and in the recognition of pathogens and secretion of antimicrobial substances. However, few studies have been conducted trying to isolate and characterize the factors involved in these effects. **Objectives:** This study aimed to find new molecules possessing activity for the stimulation or inhibition of microbial growth in the hemolymph and fat body of *Lonomia obliqua*. **Methods:** A total of 70 pupae of *L. obliqua* were selected, 45 of which were challenged with a bacterial mixture of *Escherichia coli* and *Micrococus luteus* and the remaining 25 were not challenged. After 48 h, hemolymph and fat body were extracted and subjected to solid phase extraction using Sep-Pak C18 columns, eluted in two concentrations of acetonitrile (40 and 80%). The rates obtained were subjected to liquid chromatography (HPLC), using a Jupiter C18 semi-preparative column. The fractions were assayed for stimulation or inhibition of microbial growth in liquid medium using microplates. We used for our tests the following microorganisms: *Escherichia coli*, *Micrococus luteus* and *Candida albicans*. The fractions that showed antimicrobial activity and growth were repurified by liquid chromatography using an analytical Shim-pack VP-ODS. The molecules obtained from this step were further analyzed for their biological activity. **Results and Discussion:** Three fractions were found in the hemolymph of animals not challenged, and two fractions in the hemolymph of challenged animals, which showed antimicrobial activity against all three microorganisms. For *M. luteus* and *C. albicans,* two fractions of fat body in challenged animals were found, which showed an increase in growth. We isolated seven molecules in the hemolymph and fat body of the pupae of *L. obliqua*: five of them with antimicrobial activity (hemolymph) and two with microbial growth-stimulating activity (body fat). These molecules are being analyzed by mass spectrometry for subsequent characterization.

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5.13 Identification and characterization of surface-exposed plasminogen-binding proteins of *Leptospira interrogans*

Vieira ML[1,2], Atzingen MV[1], Oliveira TR[1], Oliveira R[1,2], Andrade DM[1], Vasconcellos SA[3], Nascimento ALTO[1,2]

[1]Centro de Biotecnologia, Instituto Butantan, SP, Brasil; [2]Programa de Pós-Graduação Interunidades em Biotecnologia, [3]Laboratório de Zoonoses Bacterianas do Departamento de Medicina Veterinária Preventiva e Saúde Animal, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, SP, Brasil

Introduction: Leptospirosis is a multisystem disease caused by pathogenic strains of the genus *Leptospira*. We have reported that *Leptospira* are able to bind plasminogen (PLG) to generate active plasmin in the presence of an activator, and to degrade purified extracellular matrix fibronectin. **Objectives:** As a follow up to the previous study, we decided to screen recombinant surface exposed leptospiral proteins available in our laboratory as to their ability to bind PLG. **Methods:** We cloned, expressed in *E. coli* and purified 14 leptospiral recombinant proteins. The proteins were confirmed to be surface exposed by immunofluorescence microscopy and were evaluated for their ability to bind PLG. **Results and Discussion:** We identified eight as PLG-binding proteins, including the major outer membrane protein LipL32, the previously published rLIC12730, rLIC10494, Lp29, Lp49, LipL40 and MPL36, and one novel leptospiral protein, rLIC12238. Bound PLG could be converted to plasmin by the addition of urokinase-type PLG activator (uPA), showing specific proteolytic activity, as assessed by its reaction with a specific chromogenic plasmin substrate. The addition of the lysine analog 6-aminocaproic acid (ACA) inhibited the protein-PLG interaction, thus strongly suggesting the involvement of lysine residues in plasminogen binding. The binding of leptospiral surface proteins to PLG was specific, dose-dependent and saturable. PLG and collagen type IV competed with LipL32 protein for the same binding site, whereas separate binding sites were observed for plasma fibronectin. Our results indicate the presence of multiple PLG-binding proteins in *L. interrogans*. PLG-binding and activation through the proteins/receptors on the surface of *Leptospira* could help the bacteria to specifically overcome tissue barriers, facilitating their spread throughout the host.

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5.14 Type IV pilus: the role of PilS and PilV in atypical enteropathogenic *Escherichia coli* in interaction with epithelial cells *in vitro*

Freitas NC, Ozaki CY, Souza CS, Aires KA, Piazza, RMF
Laboratório de Bacteriologia, Instituto Butantan, SP, Brasil

Introduction: Enteropathogenic *Escherichia coli* (EPEC) have been identified as etiologic agent of diarrhea in developing and industrialized countries. The virulence of EPEC resides in its ability to promote the attaching-effacing (A/E) lesion. The various proteins involved in A/E lesion formation are encoded by genes located on the pathogenicity island called the locus of enterocyte effacement (LEE). Moreover, EPEC strains may carry a large plasmid known as the EPEC adherence factor plasmid (pEAF), which encodes the bundle-forming pilus (BFP). The EPEC pathotype was subdivided into typical (tEPEC) and atypical (aEPEC) with the basic difference being the respective presence or absence of pEAF and expression or not of BFP. This fimbrial adhesin belongs to a family of type IV bacterial pili and play an important role in the adherence of the bacteria to the enterocyte. In contrast, as aEPEC does not express BFP fimbriae, other fimbrial adhesins could be implicated in its pathogenesis. The pilin PilS and the adhesin PilV, also described as type IV pilus, contribute to plasmid conjugation, epithelial cell adherence, and adherence to abiotic surfaces of enteroaggregative *E. coli* (EAEC) isolates. **Objectives:** In this study, we evaluated the involvement of PilS and PilV proteins in the interaction of aEPEC isolates with epithelial cells in vitro. **Methods:** The presense of *pilS* and *pilV* genes was investigated in aEPEC strains by PCR. The amplified products were purified and cloned into cloning vector pGEM-T. The recombinant plasmids pGEMT-pilS and pGEMT-pilV were digested with *BamHI* and *HindIII* restriction enzymes to release the insert, which was subsequently cloned into expression vector pET28a. HEp-2 epithelial cells were used for adhesion assays, with cell-bacterial interaction of 3 and 6 h, in the presence or absence of mannose. **Results and Discussion:** The *pilS* and *pilV* genes were amplified in isolates BA558, BA956 and BA1244. Adhesion assays showed the following patterns: localized adherence in 6 h for BA558 (LA6h), non adherent for BA956 (NA) and localized-like adhesion for BA1244 (ALL). These results suggested that PilS and PilV proteins are possibly involved in these phenotypes. In the next steps, the PilS and PilV proteins will be purified to produce polyclonal sera to study the role of these proteins in the bacterial-cell interaction.

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5.15 Validation of sterility test in isolator of influenza vaccine formulated at Butantan Institute by means of bacteriostatic and fungistatic effect

Agostini Utescher CL, Orozco SFB, Cunha AA, Duboc MA
Serviço de Controle de Qualidade, Instituto Butantan, SP, Brasil

Introduction: Sterility of influenza A/H1N1 vaccine (inactivated and fragmented) formulated at Butantan Institute is done by filtering the product with a 0.45-µm membrane, rinsing this membrane with a neutralizing fluid and subsequent incubation in fluid thioglycollate medium (FTM) and soybean-casein digest media (SCM). The current standards require that all operational procedures used in quality control be validated according Current Good Laboratory Practices (cGLP). **Objectives:** The aim of this study was to determine the sensitivity of membrane filtration methodology applied to bacterial and fungal sterility testing in the influenza A/H1N1 vaccine (inactivated and fragmented) in isolators and to validate this technique under Microbiological Control Section conditions. **Methods:** This study was performed using three batches of influenza A/H1N1 vaccine (inactivated and fragmented) formulated by Butantan Institute, previously evaluated for thimerosal concentration by a spectrophotometric method. These bulks were tested according to standard methodology and membranes were rinsed with Diluent Neutralizing Pharmacopoeic fluid (DNP). After product filtration, an inoculum of viable ATCC microorganisms (10-100 CFU/mL) was added to the final portion of DNP fluid used to rinse the membrane. Fluid thioglycollate medium (FTM) was challenged with *Clostridium sporogenes, Pseudomonas aeruginosa* and *Staphylococcus aureus,* and soybean-casein digest medium (SCM) was challenged with *Aspergillus brasiliensis, Bacillus subtilis* and *Candida albicans*. Culture media were incubated for no more than 5 days at 20°C - 25°C and 30°C – 35°C (SCM and FTM, respectively). **Results and Discussion:** Characteristic growth of all microorganisms was obtained after the incubation period. The methodology applied in isolator to test the bacterial and fungal sterility of influenza A/H1N1 vaccine (inactivated and fragmented) is effective and thimerosal present in the product formulation as preservative was completely inactivated rinsing the membrane with DNP fluid. We concluded that the methodology applied detects low levels of microbial contamination, providing the public with a safe product (sterile) according to national and international standards. The sterility test may be carried out without further modifications.

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5.16 Standardization of single molecule analysis of replicated DNA (SMARD) of *Trypanosoma brucei* chromosome I

Calderano SG[1,2], Kosiyatrakul S[2], Schildkraut CL[2], Elias MC[1]
[1]Laboratório de Parasitologia, Instituto Butantan, SP, Brasil; [2]Department of Cell Biology, Albert Einstein College of Medicine, New York, USA

Introduction: DNA replication is a crucial step during the cell cycle, and until now, little is known about the DNA replication of *Trypanosoma brucei*. **Objectives:** Here, we aimed to define how replication occurs on chromosome 1 from *T. brucei* (1.85 and 3.6 Mbp). **Methods:** Single molecule analysis of replicated DNA (SMARD) allows the visualization by fluorescent microscopy of the single molecules of replicated DNA stretched on microscope slides. Using this method, it is possible to determine the numbers of replication origins, the fork direction and the DNA replication rate (Kb/min) of a DNA fragment. **Results and Discussion:** Since the technique limits the molecules analyzed to a maximal length of 500 kbp, two different approaches were developed: the analysis of chromosome 1 (1.85 and 3.6 Mbp) fragments smaller than 500 kbp and the entire chromosome 1 (1.85 Mbp). For the analysis of the fragments, DNA was digested with two different enzymes, FseI and AscI. After pulsed-field gel electrophoresis, the fragments were analyzed using specific probes, and they showed length differences compared to the prediction because the strain sequenced (*T. brucei* TREU 972) was different from that used in this experiment (*T. brucei* 427). Although the fragments were still smaller than 500 kbp, they could be identified on slides by specific probes that are being developed. In order to analyze the entire chromosome 1 (1.85 Mbp), a PFEG program will be developed to separate and extract chromosome 1 from the genome. Probes are also being developed to determine the chromosome 1 orientation (5'–3'). Once all these probes are available, chromosome 1 and the fragments will be analyzed, and then, it will be possible to know how many origins are present on chromosome 1, and also the fork direction and speed.

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5.17 Identification of bovine papillomavirus types in Brazil: co-infection with a new type and a rare variant in a dairy cow

Sakata ST[1,2], Carvalho RF[1], Mori E[2], Brandão PE[2], Richtzenhain LJ[2], Stocco RC[1], Beçak W[1]
[1]Laboratório de Genética, Instituto Butantan, SP, Brasil; [2]Laboratório de Biologia Molecular Aplicada e Sorologia, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, SP, Brasil

Introduction: Bovine papillomaviruses (BPVs) are distributed worldwide. These viruses are recognized as causal agents of several benign and malign tumors in cattle, such as cutaneous fibropapillomas, benign fibroplasias in other tissues (teats, genitals), urinary bladder and esophagus cancer. BPVs are related to severe economic losses in meat, milk and leather production. Ten different BPV types have been described and other putative types have been proposed by molecular techniques, such as PCR with generic primers and DNA sequencing. Brazil has a cattle herd of approximately 250 million, and thus the improvement of studies concerning diversity, prevalence and related clinical aspects of BPVs are very relevant. The identification of putative viral types is important for the development of vaccines. **Objectives:** Here, we describe the simultaneous presence of two BPV types in three different warts of a Holstein dairy cow with cutaneous papillomatosis. **Methods:** Wart biopsies were obtained and submitted to histological and molecular techniques. DNA was extracted for viral typing using PCR-RFLP directed to a L1 gene segment (major viral capsid protein) and subsequent sequencing. The L1 gene has taxonomic relevance due to its high degree of conservation. **Results and Discussion:** In all three lesions studied, different restriction patterns were detected which could not be identified among the profiles of any of the ten previously characterized viral types. The sequencing of the amplicons indicated the presence of a rare putative variant (BAPV-3), originally described in Japan. Furthermore, the analysis of the two other samples demonstrated in both a different viral sequence, exceeding 10% of divergence when compared to the other homologous BPVs sequences described in Genbank. It is possible to conclude that this viral sequence represents an entirely new putative type of BPV. The PCR-RFLP/sequencing procedure has been found to be highly effective for BPV identification. This approach provided the first description of BAPV-3 in Brazil and, more importantly, the identification of a possible unreported BPV type, provisionally named 01SP/BR/2009. The relevance of these findings is also related to further vaccine development procedures.

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