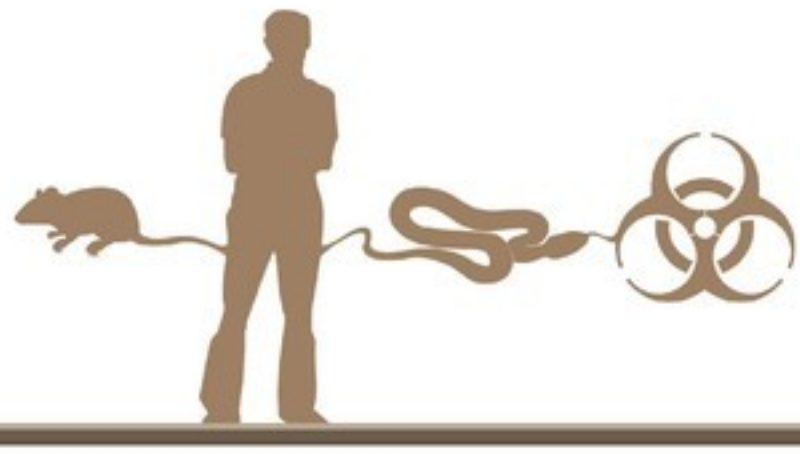
5. Microorganisms



XIV Reunião Científica Anual do Instituto Butantan
05 a 07 de Dezembro 2012, São Paulo, SP

5.01 Genes encoding autotransporter proteins of diarrheagenic *Escherichia coli* are present in typical and atypical EPEC

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Introduction: The type V secretion system is the most widespread secretion pathway for the transport of molecules across the outer membrane of Gram-negative bacteria. Members of this family have been identified in *Escherichia coli* and other Gram-negative bacteria, and are often associated with virulence functions, such as adhesion, aggregation, invasion, biofilm formation and toxicity. **Objectives:** The aim of this study was to evaluate in EPEC strains the presence of the autotransporter proteins identified in other diarrheagenic *E. coli* (DEC). **Methods:** A total of 117 EPEC isolated from cases of diarrhea (45 typical and 72 atypical) were analyzed by PCR for the presence of 17 genes encoding autotransporter proteins: *aida-I, cah, eatA, ehaABCDJ, epeA, espC, espI, espP, pet, pic, sab, sat* and *tibA*. **Results and Discussion:** With the exception of *aida-I*, *epeA* and *sab*, the other genes investigated were detected among the EPEC strains. There was a higher frequency of *ehaABCD* and *espC* genes of the enterohaemorrhagic *E. coli* (EHEC). These genes were found in both atypical and typical EPEC, as well as the *eatA* of the Shiga toxin producing *E. coli* (STEC) and *pet* genes of the enteroaggregative *E. coli* (EAEC), and *ehaJ*, in lower frequencies. The *cah*, *espI* and *espP* genes of the EHEC and *pic* of the EAEC were detected only in atypical EPEC, while *sat* of the DAEC and *tibA* of the STEC, were detected only in typical EPEC. According to the classification of typical EPEC in class 1(flagellar antigens H6 and H34) and class 2 (flagellar antigen H2), the majority of the genes investigated in this study were detected in strains belonging to typical EPEC class 1. However, *ehaAD* genes were more common in strains belonging to typical EPEC class 2, and *eatA*, *sat* and *tibA* were detected only in this group. Thus, we conclude that the majority of virulence genes investigated in this study, originally described only in specific pathotypes of *E. coli*, are also present in EPEC, especially in the subgroup of atypical EPEC.

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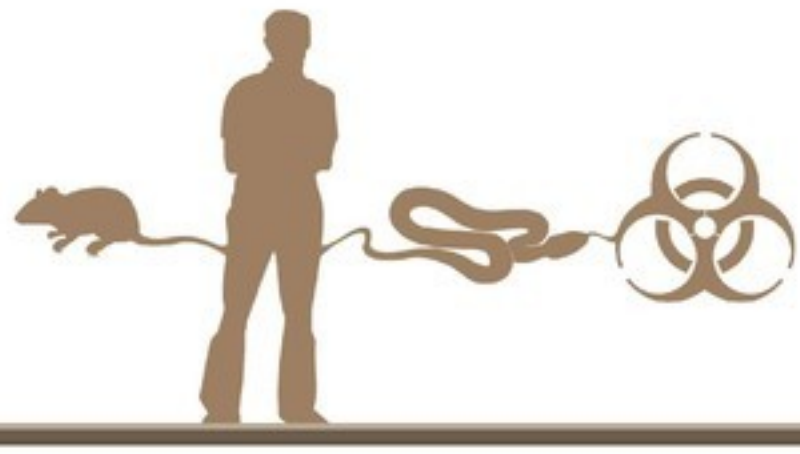

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5.02 A multiplex PCR assay for diagnosis of typical and atypical enteroaggregative *Escherichia coli*

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Introduction: Enteroaggregative *Escherichia coli* (EAEC) are very prevalent enteropathogens characterized by the expression of the aggregative adherence (AA) pattern in cultured epithelial cells. This diarrheagenic *E. coli* (DEC) pathotype is subdivided in typical and atypical, according to the presence or absence of *aggR,* respectively. Both subgroups are agents of acute and persistent diarrhea. The adherence assay using cultured epithelial cells, considered the gold standard for EAEC diagnosis, is expensive and limited to reference laboratories. PCR assays have been proposed as an alternative for EAEC diagnosis, employing more than one genetic marker, since no common genetic determinant has been found in this heterogeneous DEC pathotype. Although several EAEC virulence genes encoding adhesins, toxins, and secreted proteins are plasmid borne, a few are encoded on chromosomal pathogenicity islands, as the *aaiA-Y* operon of the prototype strain EAEC 042. **Objectives:** The aim of this study was the standardization of a multiplex PCR assay for the diagnosis of typical and atypical EAEC. **Methods:** The following EAEC genes were selected for the multiplex composition: *aggR*, *aatA* (plasmid-encoded), *aaiA* and *aaiG* (chromosomally encoded). Initially, the multiplex reaction was set up using purified DNA of EAEC prototype strain 042 as positive control and *E. coli* K12 as negative control, and the detection limit was determined. The multiplex reaction was employed to evaluate a collection of 103 EAEC strains previously characterized. **Results and Discussion:** The detection limit of the multiplex PCR assay was 125 ng of purified DNA and in this concentration the expected four genes (*aggR*, *aatA*, *aaiA* and *aaiG*) were detected. Among the 103 strains studied, a total of 78 (75.7%) were detected by the multiplex PCR. When the strains were grouped as typical and atypical, our multiplex detected all typical strains and 24 (49%) of the atypical ones. Thus, the inclusion of chromosomal genes (*aaiA* and *aaiG*) in our test represented an increase of 23.3% in the detection of EAEC. We can conclude that the proposed multiplex PCR is a good alternative for the molecular diagnosis of both typical and atypical EAEC.

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5.03 Characterization of atypical enteropathogenic *E.coli* (EPEC) outer membrane proteins profiles

Martins MBP[1], Barreto SS[1], Abreu PAE[1], Paes Leme AF[2], Piazza RMF[1], Elias WP[1], Sonobe MH[1]
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Introduction: Enteropathogenic *E. coli* has been identified as one of the main responsible agents of acute diarrhea in developing country populations. Diarrhea is still one of the most significant causes of global child mortality between 0-5 years old. **Objectives:** The goal of this study is to characterize and identify the outer membrane proteins (OMPs) of extracts derived from two strains of EPEC. **Methods:** Strains BA320 (serotype 055:H7) and BA 4013 (serotype O83:H6) were selected for this study. The OMPs were analyzed by two dimensional electrophoresis (2-DE), the first dimension by focalization of 13cm, pH range of 4-7 strips (IPGphor III, GE Healthcare) and the second by SDS-PAGE using 15% SDS-polyacrylamide gels (SE 600 Ruby, GE Healthcare). The identification was done by removing the spots from the gels and digestion with trypsin followed by mass spectrometric analysis on ESI QTOF Ultima – Waters. The resulting data were analyzed with a non-redundant protein database (NCBInr) using Mascot v3.0 engine (Matrix Science, www.matrixscience.com). **Results and Discussion:** Thirty three spots were identified with high scores for BA320 strain, allowing for the characterization of nineteen distinct proteins. For BA4013 strain, forty five spots were identified with twenty four distinct proteins. The majority of proteins were localized in the membrane, indicating the efficiency of the extraction method. Ten proteins were identical between the strains. Seven proteins were OMPs or porins (Omp A, Omp C, Omp W, Omp X, nucleoside channel receptor of phage T6 and colicin K, maltoporin and TolC). Three transporters were identified (Tsx, Vitamin B12 and fatty acid transporters). Four enzymes were detected (ATPD, phosphopyruvate hydratase, Isocitrate lyase and phosphopentomutase). Moreover, chaperonin (Groel GROES ADP7), flagellin, protection protein (DPS), one hypothetical protein and two proteins of unknown function (YgaU and YgiW) were identified. The preliminary data indicate that the majority of proteins identified have important roles in membrane permeability and at least three of them, Omp A, Omp X and flagellin are involved in the adhesion of the pathogen to host cells. Identification of proteins of unknown functions creates possibilities to gather important missing information in future.

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5.04 Role of LuxS in the regulation of virulence factors in atypical *Escherichia coli*

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Introduction: Enteropathogenic *Escherichia coli* (EPEC), a major causative agent of childhood diarrhea in developing countries, can be classified as typical or atypical EPEC. The main feature of its pathogenesis is the formation of the "attaching and effacing" (A/E) lesion in the intestinal epithelium. The proteins involved in A/E lesion are encoded by genes located in a pathogenicity island named "locus of enterocyte effacement" (LEE). Some studies suggest that LEE genes are regulated by quorum sensing, and probably the signals involved in this regulation are molecules produced by EPEC, as well as the host, the resident microbiota and transient species. To date, four quorum sensing systems were described, the ones that use autoinducer-one (AI-1) and autoinducer-3 (AI-3) are found in Gram-negative bacteria, Gram-positive use a polypeptide induction (AIP) and the system that uses autoinducer-2 (AI-2) is found in both Gram-positive and Gram-negative bacteria, and may represent an interspecific signaling system. AI-2 is synthesized by LuxS from a metabolic product of S-adenosylmethionine (SAM). **Objectives:** The aim of this study was to verify the role of LuxS/AI-2 in the regulation of virulence factors in atypical EPEC. **Methods:** We analyzed the adherence pattern and A/E lesion formation from strains O55:H7 wildtype, O55:H7 deleted in *luxS* and O55:H7 complemented with *luxS*. **Results and Discussion:** After different periods of bacteria - epithelial cells interaction (1h, 2h, 3h, 4h, 5h and 6h) no change was observed between wildtype and mutant strains. These results demonstrate that *luxS* deffective strains are fully capable of forming A/E lesion, indicating that this phenotype is independent of regulation by AI-2 and probably rely on AI-3 since its synthesis is not affected in this mutant.

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5.05 Expression of recombinant anti-intimin antibodies (scFv-intimin): a comparative analysis of different expression vectors

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Introduction: Intimin is the main virulence factor involved in the pathogenesis of enteropathogenic *Escherichia coli* (EPEC) and enterohaemorrhagic *Escherichia coli* (EHEC). The detection of EHEC and EPEC is fundamental in defining the therapeutic management of infections promoted by *E. coli*, which are still the leading cause of acute diarrhea in children and adults in many industrialized and developing countries. Antibodies are important tools in the detection of many pathogens. In our laboratory, we have obtained a monoclonal anti-intimin which was characterized as IgG2b and 1 μg of this antibody recognized 0.6 μg of purified intimin showing 1.3 x 10^{-8} M of dissociation constant, on the other hand, this monoclonal antibody showed lower reactivity with EPEC and EHEC isolates than polyclonal antibodies. **Objectives:** This fact led us to obtain anti-intimin antibody recombinant (intimina-scFv). **Methods:** The heavy and light chains were obtained from hybridomas secreting monoclonal antibodies using random primers for cDNA, these sequences were cloned into pGEMT-Easy vector and sequenced, and subsequently the chains are joined by a linker peptide. **Results and Discussion:** After synthetic scFv gene obtention the scFv was cloned into expression vectors, as pAE and pSMT3 and transformed into BL21 (DE3) *E. coli* and subjected to induction. After examining the most excellent protein expression, we observed that, both vectors directed the scFv-intimin to inclusion bodies. Thus, we choose the pAE vector to express and purify the recombinant antibody. Once purified this important tool will be used to standardize a fast and effective diagnostic method for detection of EPEC and EHEC.

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5.06 Influence of *qseC* gene in biofilm formation by atypical enteropathogenic *Escherichia coli*

Culler HF[1], Mota CM[2], Higa JS[1], Ruiz RM[1], Bueris V[1], Franzolin MR[2], Sircili MP[1]
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Introduction: Enteropathogenic *Escherichia coli* (EPEC) is one of the etiologic agents of persistent diarrhea in children. Recent epidemiological studies have shown an increase number of Atypical EPEC (aEPEC) compared to typical EPEC in number of cases, and for this reason, we can classify aEPEC as emerging pathogens. aEPEC are differentiated from typical EPEC by the absence of the EPEC *adherence factor* plasmid (pEAF) and not expressing *bundle forming pilus* (*bfp*). The adherence in epithelial cells forming microcolonies is a characteristic of EPEC, allowing biofilm formation. These are associated with bacterial persistence and resistance to antibiotics. Several atypical EPEC adherence patterns form biofilms on abiotic and pre-fixed cell surfaces. Various mechanisms in *E. coli* are controlled by Quorum Sensing (QS), including the expression of virulence factors and biofilm formation. QS is a signaling system which gives some bacteria the ability to respond to chemical molecules, called autoinducers (AI). Bacteria are able to produce, release, detect and respond to these signaling molecules and when these autoinducers reach a critical concentration due to increase cell density, there is an activation of transcription factors, altering gene expression. These molecules can be divided into four categories, amino acids and small peptides, autoindutor-1 (AI-1); autoindutor-2 (AI-2) and autoindutor-3-(AI-3). Quorum sensing *Escherichia coli* regulator C (QseC) receptor is responsible for detecting the IA-3 and is also able to detect adrenaline and noradrenaline. **Objectives:** The objective of this study was to investigate the role of *qseC* in biofilm formation by aEPEC. **Methods:** *qseC* mutants were obtained by homologous recombination, and analyzed for biofilm formation through a period of 24 hours of incubation using the methodology of crystal violet using preconditioned medium and Luria-Bertani broth. **Results and Discussion:** Our preliminary results with 24 hours of incubation indicate that there was no influence in biofilm formation between wildtype and mutant strains.

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5.07 Influence of *sdiA* gene in biofilm formation by atypical enteropathogenic *Escherichia coli*

Culler HF[1], Mota CM[2], Higa JS[1], Ruiz RM[1], Bueris V[1], Franzolin MR[2], Sircili MP[1]
[1]Laboratory of Genetics, Instituto Butantan, SP, Brasil; [2]Laboratory of Bacteriology, Instituto Butantan, SP, Brasil

Introduction: The atypical *Escherichia coli* enteropathogenic (aEPEC) are capable to cause the lesion A/E and they do not transport the pEAF. The adherence in epithelial cells forming microcolonies is a characteristic of EPEC, allowing biofilm formation. These are associated with bacterial persistence and resistance to antibiotics. Microbial biofilms are defined as complex communities formed by microorganisms adhered to surfaces embedded in an exopolysacharidic matrix. Various mechanisms in *E. coli* are controlled by Quorum Sensing (QS), including the expression of virulence factors and biofilm formation. QS is a signaling system which gives to some bacteria the ability to respond to chemical molecules, called autoinducers (AI). Bacteria are able to produce, release, detect and respond to these signaling molecules and when these autoinducers reach a critical concentration due to increased cell density, there is an activation of transcription factors, altering gene expression. These molecules can be divided into four categories, amino acids and small peptides, autoindutor-1 (AI-1); autoindutor-2 (AI-2) and autoindutor-3-(AI-3). Suppressor of cell division inhibition A (SdiA) receptor, encoded by the *sdiA* gene is responsible for detection of AI-1 and indole, but *E. coli* does not produce molecules of AI-1 type, thus detecting AI-1 molecules produced by other bacteria. **Objectives:** The objective of this study was to investigate the role of *sdiA* in biofilm formation by aEPEC. **Methods:** *sdiA* mutants were obtained by homologous recombination, and analyzed for biofilm formation for 24 hours of incubation using the methodology of crystal violet with preconditioned medium and Luria-Bertani broth. **Results and Discussion:** Our preliminary results with incubation in periods of 24 hours indicate that there was no influence in biofilm formation between wild-type and mutant strains.

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5.08 Expression of CXCL13 mRNA in liver and lung of mice infected with pathogenic *Leptospira* spp

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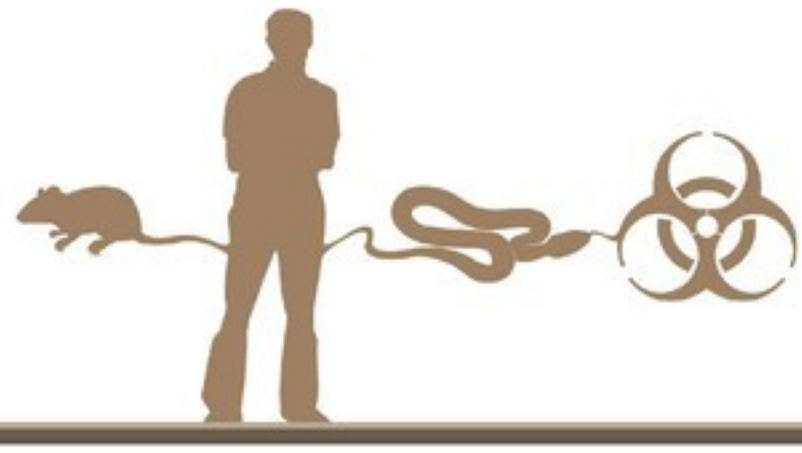
Introduction: Leptospirosis is a worldwide zoonosis caused by pathogenic *Leptospira*. Pathogenesis of *Leptospira* spp infection in humans is mainly observed in lungs, kidneys and livers. Although several components of the pathogen and host interaction are been identified, the molecular mechanisms of this infectious disease are still poorly understood. Injury promoted by microbial factors and cytokines and chemokines produced in response to infection have been proposed to be involved in pathogenesis of leptospirosis. Recent reports have pointed to a broader role to the homeostatic chemokines, including modulation of inflammatory responses in lymphoid and non-lymphoid tissue. The chenmokine CXCL13 is crucial for B cell trafficking. It has also been related to T cell and monocyte activation and apoptosis. **Objectives:** In the present study, the expression of the gene of chemokine CXCL13 in liver and lung of infected mice was investigated. **Methods:** Mouse strain C3H/HeJ, C3H/HePas and BALB/c mice were infected intraperitoneally with $1x10^7$ bacteria the virulent *Leptospira interrogans* serovar Copenhageni. Three mice for each mouse strain (n=12) were sacrificed on days 1, 3, 5 and 7 after infection. Three un-infected animals of each strain were sacrificed on day 0 as control. Total RNA was isolated from tissues, quantified and used to prepare cDNA. The expression of CXCL13 chemokine and GAPDH was detected by Real-Time PCR, and relative expression of the genes was calculated by $2^{-\Delta\Delta}$CT method, using GAPDH gene to normalize. **Results and Discussion:** Elevations on the levels of CXCL13 mRNA expression were observed in liver of three strains of mice and in lung of C3H/HeJ mice. The levels of CXCL13 mRNA in liver were higher in C3H/HePas and BALB/c than in C3H/HeJ strain. In the two strains more resistant to leptospirosis higher levels of the CXC13 chemokine were detected at third day after infection, whereas in the strain susceptible to leptospirosis, C3H/HeJ, higher levels were measured at fifth day in liver and seventh day in lung. Our results indicate that the expression of CXCL13 can vary greatly, depending on the tissue, mouse strains and time post-infection. The preliminary results indicated that an increasing in CXCL13 and the premature production in organs of C3H/HePas and BALB/c strains could contribute to the strength of these animals against leptopsirosis. On the other hand, the later production in liver and lung of C3H/HeJ can contribute to the severity and progression of leptospirosis. This study is important to provide information for proper vaccine development and for further elucidation of the mechanisms of pathogenesis in leptospirosis, thus contributing to the development of more effective treatments and prevention of the disease.

Supported by: FAPESP, CNPq and Fundação Butantan



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5.09 AI-3/Epinephrine/Norepinephrine *Quorum Sensing* system regulates atypical enteropathogenic *Escherichia coli* virulence

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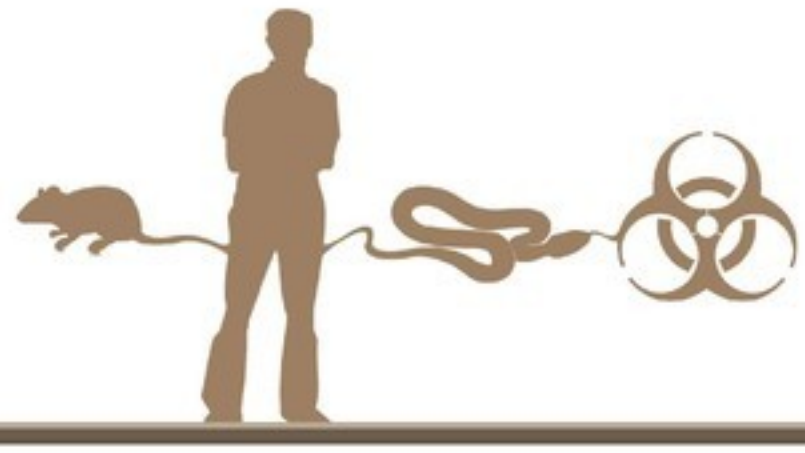
Introduction: The Attaching and Effacing (A/E) lesion, the virulence feature of atypical enteropathogenic *Escherichia coli* (aEPEC), is forming due the activation of genes located in a pathogenicity island called LEE (*locus of enterocyte effacement)*. The transcription of LEE is subject to regulation by innumerous factors, including *Quorum Sensing*. Four systems of *Quorum Sensing* have been described, including the autoinducer 3 (AI-3) system. This system involves the detection of AI-3 and the mammalian hormones epinephrine and norepinephrine through the sensor histidine kinase QseC and activates the transcripition of virulence genes. **Objectives:** The objective of this study was to determine the effects of this *Quorum Sensing* system in aEPEC virulence. **Methods:** aEPEC strain lacking *qseC* gene was generated using *lambda-red* recombination method and analyzed in the presence and/or absence of epinephrine by phenotypical test (FAS) and transcriptional tests (DNA *Microarray* and qRT-PCR). The expression and secretion of translocated factor EspA was analyzed by *Immunoblot*. **Results and Discussion:** FAS test has shown a decrease in the ability to form A/E lesion of mutant strain. The global assessment of QseC gene regulation in aEPEC has shown 491 pathogen-specific genes decreased in their transcriptional levels, while in the presence of epinephrine, 129 pathogen- specific genes were decreased. qRT-PCR of LEE operons were performed and has shown that *ler* gene transcription is highly decreased in the mutant strain, but highly increased in the presence of epinephrine while the other operons have the transcription levels decreased in the presence of epinephrine. The production of EspA was not affected by the mutation, but the secretion was greatly impaired. Transcription levels of LEE operons suggested that they are under direct or indirect QseC regulation in the epinephrine dependent manner in aEPEC and that QseC is not the only receptor for epinephrine in this strain. The decrease in the ability to form A/E lesion and the transcriptional levels of pathogen-specific genes in the mutant shown that QseC has a key role in pathogenicity of aEPEC. Taken together, these results lead us to propose a model for virulence regulation of aEPEC by the AI-3/epinephrine/norepinephrine system. Because it is a system widely used by pathogens to regulate temporally and energetically the expression of virulence factors, it can be exploited to develop new therapeutic and prophylactic approaches to fight infections.



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5.10 Influence of commensal bacteria in biofilm formation from atypical enteropathogenic *Escherichia coli*

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Introduction: *Escherichia coli* (EPEC) is one of the leading causes of diarrhea among children in developing countries. Atypical EPEC (aEPEC) does not harbour the EAF (EPEC adherence factor) plasmid and became the most common bacterial agents in our environment. One of EPEC characteristics that allow the formation of biofilms is the adherence in epithelial cells forming microcolonies, which are associated with bacterial persistence and resistance to antibiotics. Quorum sensing is involved in many mechanisms of gene regulation in *E. coli*, including some virulence genes, and biofilm formation. Some bacteria can recognize signals produced by other bacterial species. **Objectives:** The aim of this study was to verify the influence of commensal bacteria present in the intestinal microbiota in biofilm formation from aEPEC in co-culture experiments, and using pre-conditioned media (PCM), which may contain these signals. **Methods**: Biofilm formation from aEPEC strains O55:H7 and ONT:H25 were analyzed using the colorimetric crystal violet assay for a period of 24h, 48h and 72h, using four different media. We tested LB, DMEM supplemented with 0.4% glucose, *E. coli* broth and PCM, prepared using commensal strains of *E.coli*, *Klebsiella pneumoniae* and both aEPEC strains. **Results and Discussion:** The results showed that there were no influence on biofilm formation using PCM, but we noticed a difference when comparing the control using only the culture medium. In addition, we noticed reduced biofilm formation when the strains were grown in *E. coli* and *K. pneumoniae* PCM. The PCM can contain these signals, which would allow the difference in biofilm formation, suggesting that molecules produced by intestinal bacteria are recognized by aEPEC, demonstrating that there is an inter-specific interaction between commensal bacteria and pathogenic strains.

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5.11 scFv antibody fragments against stx1 toxin for its use in immunodiagnostic tests of Shiga toxin-producing *Escherichia coli*

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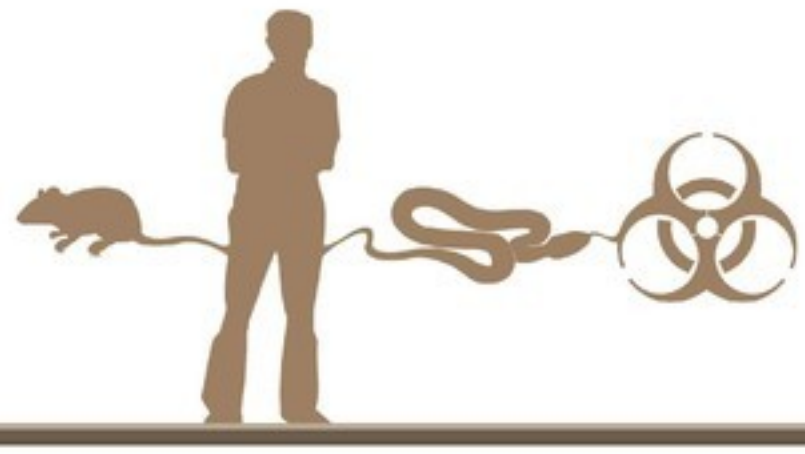
Introduction: Shiga toxin-producing *Escherichia coli* (STEC) are recognized agents of hemorrhagic colitis/hemolytic uremic syndrome. The diagnosis of diarrheal disease caused by STEC is performed using cells cultured to detect and confirm the production of Stx1/Stx2 toxins and is considered the standard test, however requires specialized work, takes time and it is expensive, which makes it unsuitable for laboratories routine diagnostic. Therefore, the development of cheap and fast diagnostic methods for these important enteric pathogens, such as immunodetection, is a need for our reality. **Objectives:** This work aimed the production of recombinant antibodies against the Stx1 toxin to be used in the immunodiagnosis of STEC strains. **Methods:** The variable heavy and light chains sequences were obtained from cDNA of the hybridomas secreting antibodies against the Stx1 mRNA. This sequences was analyzed for CDRs and confirmed by sequencing and alignment with mouse germinal IgG. The sequence obtained was used as template for synthetic gene design. The gene was cloned in pAE expression vector and the purification was performed in nickel affinity chromatography under denaturing conditions. The refolding was done using buffers with decreasing urea concentrations, and after that the recombinant protein quantified by BCA kit. The functionality of the scFv was confirmed by ELISA. **Results and Discussion:** The chains CDRs were identified by aminoacids analysis and was confirmed it is possible functional chains after the alignment with mouse germinal IgG. The cloning was successfully obtained and confirmed by restriction analysis and sequencing. Expression tests was made in 2YT broth and the recombinant antibody was detected in the insoluble fraction, the denaturing condition was required to obtain and purify the antibody, after refolding the scFv yield was 7,9 mg/L. The recombinant antibody was capable of recognize the Stx1 toxin by ELISA up to 1:1000. Obtaining these recombinant antibodies is a promising tool for the rapid diagnosis of this pathogenic strains, as these tend to be more sensitive and specific than monoclonal antibodies and, in addition, their production are faster and cheaper.

Supported by: FAPESP



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5.12 Interaction of spirochetes of the genus *Leptospira* with human vitronectin

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Introduction: Leptospirosis is an infectious disease caused by spirochetes from the genus *Leptospira*. It is transmitted by the contact of abraded skin or mucous membranes with water or soil contaminated with urine from reservoir animals, such as rodents. The disease constitutes a major public health problem in developing countries. Pathogenic leptospires efficiently colonize target organs after penetrating the host. Their invasiveness is attributed to the ability to evade the innate host defense, notably the complement system, which constitutes the first line of host defense, playing a crucial role in early recognition and elimination of leptospires. It comprises more than 30 proteins, among which the glycoprotein vitronectin, involved in the regulation of the terminal pathway of complement. **Objectives:** The present work aimed to evaluate the interaction of vitronectin with different *Leptospira* species as a mechanism of immune evasion and to identify leptospiral surface proteins involved in this interaction. **Methods:** Leptospires were cultured in EMJH medium at 29ºC under aerobic conditions. They were centrifuged and the bacterial pellets were incubated with human serum as a source of vitronectin. After successive washes, whole cell lysates were subjected to Western blot (WB) with anti-vitronectin. Binding of leptospiral outer membrane proteins with vitronectin was assessed by WB with overlay and ELISA. In this case, proteins were immobilized in microtiter plates and, after blocking, purified vitronectin was added. Binding was detected with specific antibodies. **Results and Discussion:** All *Leptospira* species interacted with human vitronectin and we identified five proteins involved in this interaction. Of special interest is a surface protein called LcpA, previously shown by our group to bind the complement regulators C4b Binding Protein and Factor H. The C-terminal domain of LcpA seems to be responsible for this interaction. The acquisition of vitronectin by leptospires has not been reported before, and the characterization of proteins that interact with host molecules may contribute to the development of therapeutic strategies to fight the disease.

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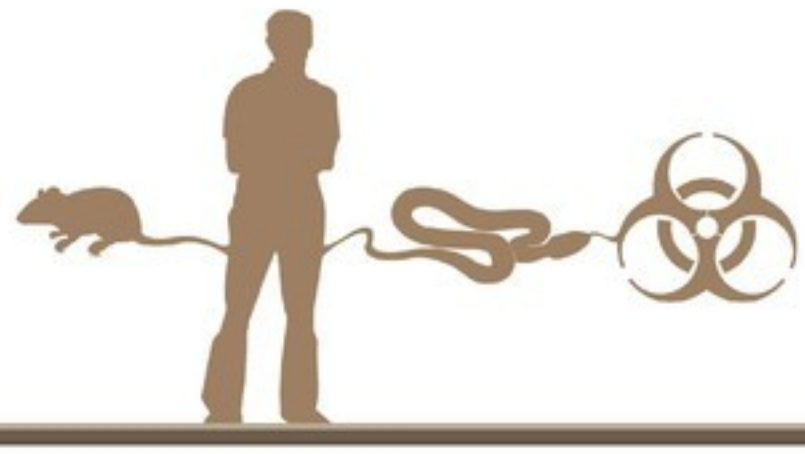

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5.13 Pellicle formation on tubes by atypical enteropathogenic *Escherichia coli* in different growth conditions and research of fimbriae

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Introduction: EPEC has been characterized as the main bacteriological agent of acute diarrhea in children from developing countries, predominating atypical EPEC. Biofilms are structured communities of microorganisms enclosed in self-produced polymeric matrices attached to biotic or abiotic surfaces. The biofilm formation, such as pellicle and flock formation, is associated with bacterial persistence and antimicrobial resistance. **Objectives:** Verify the influence of different growth conditions in the adherence of aEPEC on glass tubes, in addition to research the production of exopolysaccharide (EPS) and the presence of *crl*, *csgA* and *fimH* genes, besides the expressions of *curli* and type I fimbriae. **Methods:** 23 strains were assayed to pellicles formation in glass tubes at 26°C and 37°C for 48 hours with shaking, using Luria Bertani broth (LB), Luria Bertani broth without NaCl (LB without NaCl), *E. coli* broth and minimum M9. The putative production of EPS was verified by centrifugation. The presence of *csgA*, *crl* and *fimH* genes was detected by PCR. The expression of *curli* and type I fimbriae were verified after growth in Congo Red agar and agglutination assay with *Sacharomyces cerevisae* respectively. **Results and Discussion:** The biofilm formation on tubes surface at 26°C were positive for the majority of the strains in all broths, in opposite of the strains growth at 37°C, which gave less than 15% of positivity, pointing the importance of the permanence of the strain in the environment, representing an reservoir. Almost 100% of the strains in *E. coli* broth formed pellicles at both temperatures, as well as 95.6% of the strains growth in LB broth; 91.3% in LB broth without NaCl and 39.1% in M9 at 26°C. All strains presented *crl* gene and 26% were positive to *csgA* gene. However, only 37.4% expressed *curli* fimbriae. Only 17.4% produced EPS in the studied conditions. 95.6% of the strains presented the *fimH* gene, whereas the type I fimbriae expression occurred in 74%. The capacity of biofilm formation could be better demonstrated by employing the culture medium which is closest to the human gastrointestinal tract (*E. coli* broth), indicating the high resistance potential of these strains in the environment and in the host. Despite the relative expression of the two types of fimbriae, not all strains formed biofilm in other growth medium, indicating that another fimbriae and even adhesins could influence the pellicle formation in aEPEC, being necessary to seek other factors that can contribute to this process.

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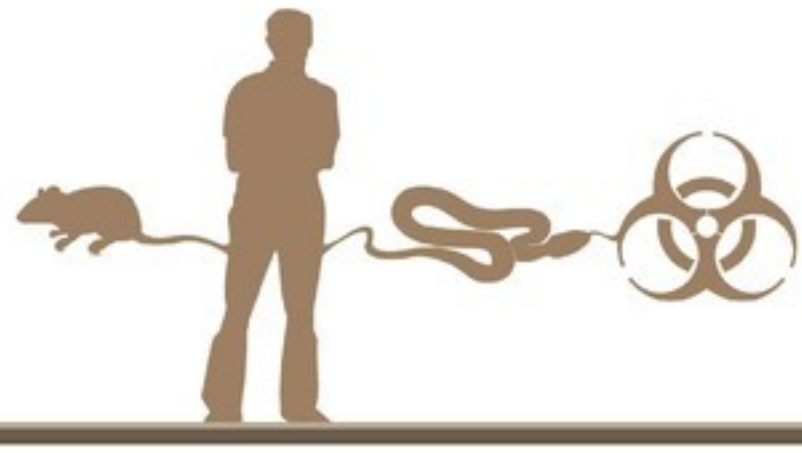

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5.14 Immunodiagnostic tool for enterotoxigenic *Escherichia coli* (ETEC): optimization of the pSMT3 ScFv-LT expression

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Introduction: Enterotoxigenic *Escherichia coli* (ETEC), one of the pathotypes of diarrheagenic *E. coli,* that produces heat-labile toxin (LT) and heat-stable (ST), is an important pathogen involved in diarrhea of children under five years old and tourists traveling in endemic areas. The quick and effective identification of the diarrhea contributes to the correct treatment of the infected patients. Genetic engineering has been used to obtain recombinant single chain antibodies (ScFv) on a large scale with low cost, and maintaining their functional properties. To use these constructions as tools for the immunodiagnosis it must be standardized conditions that favor the expression of ScFv. **Objectives:** This work aims to optimize the expression of the scFv – LT fragment from ETEC cloned into the vector pSMT3, which fuses an ubiquitin (SUMO) at the cloned protein in order to improve its solubility. **Methods:** The construction used in this study was obtained in a previous work and sequenced to identify the CDRs. To optimize the expression, different bacterial host cells were cultivated in LB, M9 and 2YT medium, with or without addition of 1% glucose at temperatures of 20, 30 and 37 °C. For induction, we used different concentrations of IPTG, without shaking, or ranging from 150 to 200 rpm for 2, 4 and 16-18 hours. Bacterial cells were disrupted using ultrasonic homogenizer SonoPuls Bandelin and the soluble fractions were analyzed by SDS/PAGE. **Results and Discussion:** The CDRs of the heavy and light chain were successfully identified. No chain mutations were found after cloning. The highest expression of the recombinant antibody was obtained in *E. coli* BL21 (DE3) cultivated in M9 medium with 1% glucose induced with 1 mM of IPTG at 30 °C for 16-18 hours at 150 rpm, and a part expressed in its soluble form. Our results are encouraging, since the consecutive changes of media and growth conditions favored the induction and expression of ScFv – LT on the soluble fraction, which can be obtained in higher yield, increasing its potential use as a tool for the diagnosis of this important group of pathogens.

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5.15 Genetic diversity of Parainfluenza virus 1, 2 and 3, identified from samples taken at the University Hospital of São Paulo University, during 1995 to 2005

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Introduction: Human parainfluenza viruses (HPIVs) are important cause of lower respiratory tract infections in infants, young children and immunocompromised patients, and have a worldwide distribution. In paediatric population the characteristic illness associated with HPIV-1 and -2 is laryngotracheobronchitis, but it can also be responsible for upper respiratory tract infection and pharyngitis, whereas HPIV-3 is also associated with pneumonia and bronchiolitis. Sequencing studies involving encoding genes fragments of HN or F proteins aim to obtain data to allow the understanding of the circulation pattern and evolution of these viruses, generating important information for both diagnosis and for vaccine development. **Objectives:** The aim of this study was to carry out the molecular analysis of the fragment of the HN gene of the parainfluenza 1, 2 and 3. **Methods:** Nasopharyngeal aspirates from 2152 infants and children under five years old, hospitalized at the University of São Paulo Hospital (HU-USP) with acute respiratory illness were collected from 1995 to 2005. The detection of HPIV 1, 2 and 3 were performed by multiplex RT-PCR using specific primers to HN gene, labeled with FAM. The fragment of HN gene (approximately 480 pb from C terminal region) was amplified, sequenced and, subjected to phylogenetic analysis. **Results and Discussion:** A total of 6% (n=135) samples were positive for one of the HPIV, and the HPIV-3 was the most frequent virus detected during all years studied, corresponding to 80 % (108/135) of positive cases, followed by HPIV-1 with 15 % (20/135) and HPIV-2 with 7 % (10/135). The positivity among the years studied ranged from 1 % (1/195) in 1996 to 18 % (24/154) in 1999. Most mutations observed in HPIV-1 and HPIV-3 were silent in all HPIVs, however, some amino acids alterations in conserved areas, verified in HPIV-3 and HPIV-2, and alterations in N-glicosilation sites were observed. Phylogenetic analysis showed a lower variability in the HPIV-1 samples, just genotype C was present, and HPIV-3. The single sample of HPIV-2 showed a higher variability compared to prototype sample, in which was found that 70 % of the changes were not synonymous.

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5.16 The influence of Hfq on the adherence and A/E lesion formation in a O55:H7 atypical EPEC strain

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Introduction: The term atypical EPEC is used to define enteropathogenic *Escherichia coli* strains that do not harbours the "EPEC adherence factor" (EAF) plasmid. The main feature of EPEC pathogenesis is the formation of the "attaching and effacing" (A/E) lesion in the intestinal epithelium. The genes responsible for this phenotype are contained in a pathogenicity island termed the "Locus of Enterocyte Effacement (LEE). LEE genes can be regulated by Quorum Sensing, a process of bacterial cell-to-cell communication involving the production and detection of extracellular signaling molecules called autoinducers. Quorum Sensing regulation by small RNAs (sRNA) is well established in *Vibrio cholerae*, where the sRNA and the chaperone Hfq form a complex called Qrr (Quorum Sensing regulatory RNA), that act on LuxR increasing or decreasing its production, and altering biofilm formation and virulence factors expression. To date, there are no studies on the involvement of Hfq regulation in atypical EPEC. **Objectives:** The aim of this study was to analyze the ability to adhere to epithelial HEp-2 cells and the capacity to form A/E lesion, from an Hfq mutant atypical EPEC strain belonging to O55:H7 serotype. **Methods:** Hfq mutants were obtained by allelic recombination using the λ Red recombination system. **Results and Discussion:** The mutant strain showed reduced adherence to HEp-2 cells. Furthermore, fluorescence actin staining (FAS) test showed a reduction in the capacity to form A/E lesion.

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5.17 Comparative analysis of anti-EspA antibodies for the diagnosis of *Escherichia coli* (EPEC) strains and enterohemorrhagic *Escherichia coli* (EHEC)

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Introduction: Enteropathogenic *Escherichia coli* (EPEC) and enterohemorrhagic *Escherichia coli* (EHEC) are important pathogens causing significant morbidity and mortality worldwide. The virulence of EPEC and EHEC 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) in which the secreted effectors protein (Esps) of type III secretion system are included. Among the proteins secreted of EspA is a promising target for the diagnosis of EPEC and EHEC. **Objectives:** Thus we raised polyclonal and monoclonal antibodies against this protein and compare the use of these antibodies for EspA detection. **Methods:** Nine K0 hybridoma clones (3G4, 3E12, 2H1, 4H4, 3B8, 2B12, 3C12, 2H9 and 2H5) producing anti-EspA antibodies were cultured and culture supernatants were collected, as well as the rabbit anti- EspA IgG-enriched fraction were tested by ELISA with nine typical EPEC, nine atypical EPEC, eight EHEC and four LEE negative *E. coli* isolates. The obtained absorbances were analyzed statistically comparing tEPEC, aEPEC and EHEC isolates versus negative LEE *E. coli* using the non-parametric Student *t* test. **Results and Discussion**: The hybridoma 3G4 showed significantly variance difference tEPEC only ($p=0.0293$) and EHEC ($p=0.0233$), 3E12 to tEPEC ($p=0.0156$) and aEPEC ($p=0.0229$) 2H1, 4H4 and 2B12 showed no variance between the groups, for tEPEC 3B8 ($p=0.0415$). However, the 2H5 hybridoma showed significantly variance difference for tEPEC, aEPEC and EHEC ($p<0.0001$, $p=0.0001$ and $p<0.0001$, respectively), 2H9 ($p=0.0013$, $p=0,0443$ and $p=0.0042$, respectively) and 3C12 ($p<0.0001$, $p<0.0001$ and $p<0.0001$, respectively). The polyclonal antibody variance showed no significantly different for both EPEC and EHEC. Hybridomas 3C12, 2H5 and 2H9 proved to be better tools for diagnosis of EPEC and EHEC than the other hybridomas and IgG enriched fraction of anti- EspA polyclonal antibody.

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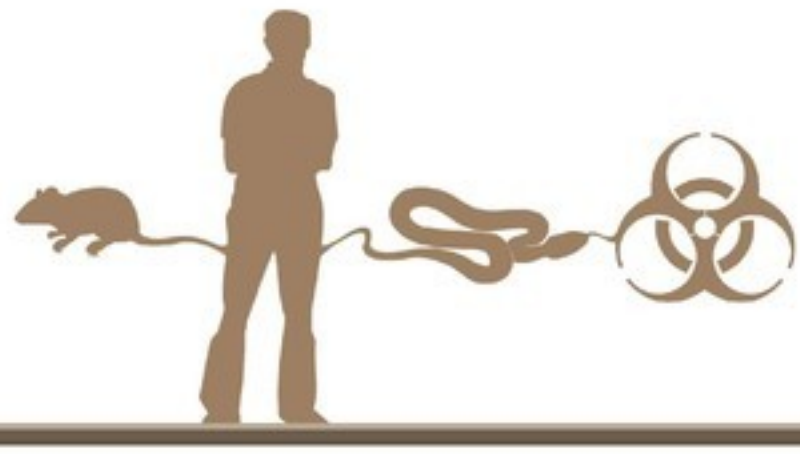

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5.18 Mapping of Leptospiral complement protein A (LcpA) domains involved in the interaction with the human complement regulators C4b Binding Protein and Factor H

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Introduction: Leptospirosis is a spirochetal disease caused by pathogenic members of the genus *Leptospira*. After penetrating the host, leptospires have the ability to disseminate and to trigger a specific immune response. Over the past few years, our group has focused on the identification of immune evasion mechanisms presented by pathogenic leptospires. Recently, we have identified an outer membrane protein, named LcpA, capable of interacting with the human complement regulators C4b Binding Protein (C4BP) and Factor H (FH). **Objectives:** In order to map the LcpA region involved in the interaction with these complement regulators, we cloned, expressed, purified and characterized three fragments of this protein corresponding to its N-terminal, intermediate and C-terminal domains. **Methods:** The fragments were amplified by PCR using *L. interrogans* serovar Copenhageni L1-130 DNA as a template, and were cloned into the pAE expression vector. The recombinant His-tagged proteins were purified by nickel-affinity chromatography. The interaction with C4BP and FH was assessed by Western blot with overlay using human serum as a source of both complement regulators. **Results and Discussion:** All three fragments were properly expressed and purified. According to our data, the C-terminal domain mediates interaction with both FH and C4BP. It seems that the binding site implicated in the interaction with C4BP comprises the last four amino acids of LcpA. The immunogenicity and vaccine potential of this protein is being evaluated in a hamster model.

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5.19 A new tool for enterotoxigenic Escherichia coli (ETEC) diagnosis: preparation and characterization of recombinant monoclonal antibodies against the Heat-Stable toxin (scFv – ST)

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Introduction: *Escherichia coli* associated with gastroenteritis are known as diarrheagenic *E. coli*. Amongst them, the enterotoxigenic *E. coli* (ETEC) is responsible annually for c.a. of 400 million diarrhea episodes and 700,000 of children deaths under five years and also the main cause of the "traveler's diarrhea". The identification of ETEC has been done by detection of its major virulence factors: the heat-labile enterotoxin (LT) and heat-stable (ST) using molecular biology techniques or immunoassays. When compared to other detection methods, immunoassays have several advantages they are rapid and easy tests to be performed, always showing high specificity and sensitivity. Although the monoclonal antibodies present excellent characteristics as consistency and specificity, and can be produced without limit, its production requires the use of cell culture and specialized extensive involvement of time and labor. **Objectives:** As alternative, recombinant antibody can be engineered, which is the aim of this study. **Methods**: For this approach, we start from a synthetic gene, codon-optimized for expression in *Escherichia coli*. This gene was amplified in the cloning vector pGEM-T Easy and subcloned into expression vector pET28a. BL21(DE3) *E. coli* cells were transformed with the recombinant plasmid and induced with IPTG in 2YT medium for its expression. **Results and Discussion:** By SDS/PAGE and immunoblotting we observed that the insoluble fraction contained a large amount of antibody fragment. Therefore it was purified in affinity nickel-chromatography column in a high-pressure system (AKTA) in urea presence, followed by a refolding step. The concentration of antibody was measured and purity was analyzed by SDS/PAGE. Further the characterization was performed through functionality analysis testing by ELISA and immunofluorescence. Our results showed that the molecule is functional and no reactivity with the negative controls was observed. Moreover, it presented stability when stored at 4 °C. As the test reactivity to strains of ETEC, the scFv showed reactivity to both the porcine and human samples, thus showing it as promising tool for use in the diagnosis of ETEC trough ST detection.

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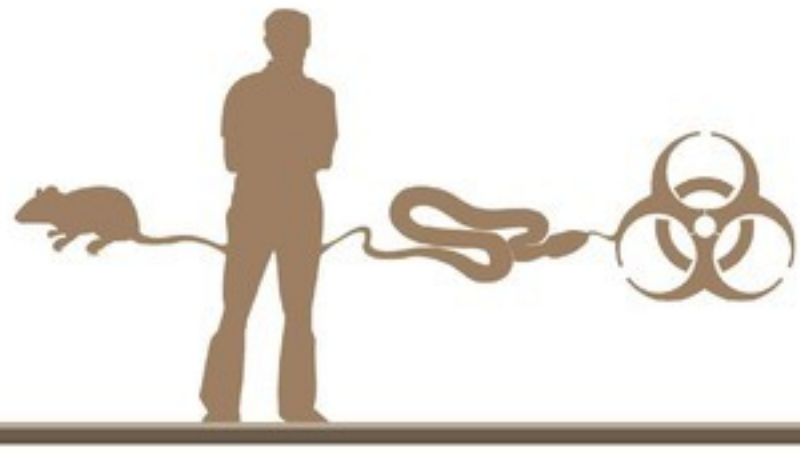

5.20 *Leptospira* interaction with plasminogen/plasmin system

Vieira ML[1,2], Alvarez-Flores MP[3], Chudzinski-Tavassi AM[3], Kirchgatter K[4], Romero EC[5], de Morais ZM[6], Vasconcellos SA[6], Nascimento ALTO[1,2]
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Introduction: Leptospirosis is a globally important zoonosis, caused by pathogenic bacteria of genus *Leptospira*. Despite its importance, the molecular mechanisms for leptospiral pathogenesis and virulence remain poorly understood. After adhesion to eukaryotic cells and extracellular matrix (ECM), the bacteria may reach the circulation in order to colonize target organs. The proteolytic activity is crucial as it allows tissue disruption and penetration. However, as the majority of pathogenic bacteria, *Leptospira* lack proteases capable of degrading these structural barriers. To overcome this absence, many pathogens evolved the ability to interact with the host's plasminogen/plasmin (PLG/PLA) system in order to acquire proteolytic activity. **Objectives:** Investigate and characterize leptospiral interactions with PLG/PLA system. **Methods:** Virulent and attenuated leptospires were assayed for the ability to bind PLG and acquire PLA activity. Recombinant leptospiral proteins were analyzed for their PLG-binding properties. The effect of leptospiral contact with endothelial cells was assessed as PLG activators and proteases contents. Sera from leptospirosis individuals were also evaluated. **Results and Discussion:** *Leptospira* capture PLG on the outer surface, which is converted to PLA by host's activators. Several PLG binding proteins were identified in *Leptospira*, some of them apparently differentially expressed in highly virulent conditions, suggesting the importance of this mechanism for pathogenesis. The surface-associated PLA degraded ECM components, facilitating the penetration through endothelial cells that may enhance the dissemination within the host. The PLA bound to the leptospires contributed to the immune evasion, by decreasing IgG and C3b depositions, thus increasing bacterial survival by complement attack. Leptospires were shown to stimulate an imbalance in the fibrinolytic and proteases systems regulation by increasing the expression of PLG activators and matrix metaloproteases (MMPs) by endothelial cells. Furthermore, confirmed leptospirosis patients showed higher levels of circulating PLG activators and MMPs, which is more pronounced at the beginning of the infection. These studies revealed a novel proteolytic activity used by the leptospires to enhance penetration, dissemination and immune evasion within the hosts, thus contributing to the comprehension of the molecular aspects of leptospirosis infection.

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