Marcadores moleculares e Técnicas de C. Molecular

Sibele Borsuk

sibele@ufpel.tche.br / sibeleborsuk@gmail.com

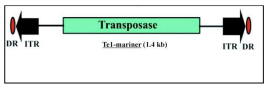
Caracterização Molecular

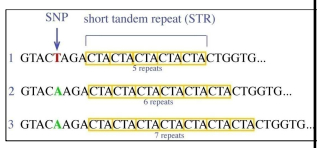
EVOLUÇÃO DOS GENOMAS, DNA É INSTÁVEL

Elementos Moveis e seqüências repetidas de DNA

Mudaram a idéia de que os genomas são unidades estáticas

Transposon/ seqüência de inserção **SNPs, STRs**





Caracterização Molecular

A correta identificação e caracterização de microrganismos é de extrema importância prática em:

- Diagnóstico - Terapia
- Epidemiologia – Rastreabilidade
- Biotecnologia
- **Vacinologia**
- Estudos de Filogenia e evolução

Caracterização Molecular

- Métodos tradicionais usados na discriminação de microrganismos:
  - Fenotípicos:
    - Testes bioquímicos
    - Testes sorológicos
    - Testes com bacteriófagos
    - Microscopia eletrônica - morfologia
  - Genotípicos:
    - Testes baseados no DNA e RNA

Trypanossoma cruzi

Isoenzimas

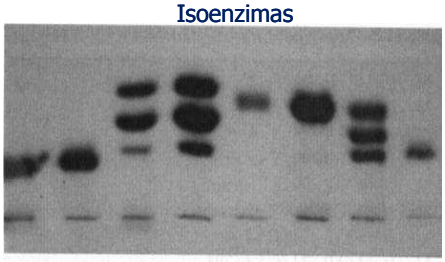


Fig. 15.6 Isoenzyme profiles of *Trypanosoma cruzi* isolates by starch-gel electrophoresis.

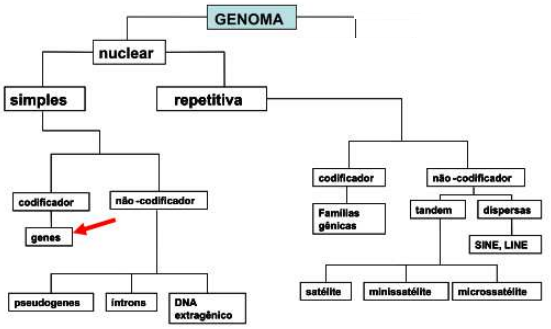
Caracterização Molecular

Métodos Fenotípicos

Desvantagens

- **tempo**
- **laborioso**
- **interpretação**
- **depende da fase de desenvolvimento**
- **padronização (antisoros)**
- **sensibilidade e especificidade**

Marcadores moleculares



Marcadores moleculares

DNA Repetitivo

- Microsatelites ou Simple Sequence Repeats (SSRs)
  – Mono-, di-, tri- and tetra-nucleotideos repetidos
- Minisatellites or Short Tandem Repeats (STRs)
  – 5-10 bp repertidos
- Variable Number of Tandem Repeats (VNTRs)
  – 14-100 bp repertidos

Marcadores moleculares

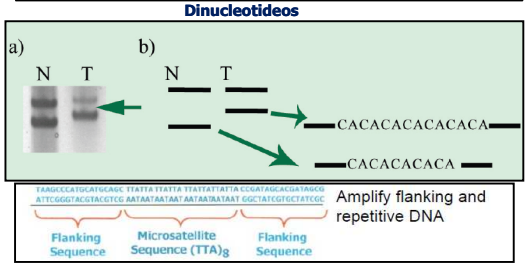
- Genes Nucleares
- Sequencias de Inserção (IS)
- SNPs (Single Nucleotide Polymorphism)

Microsatélites

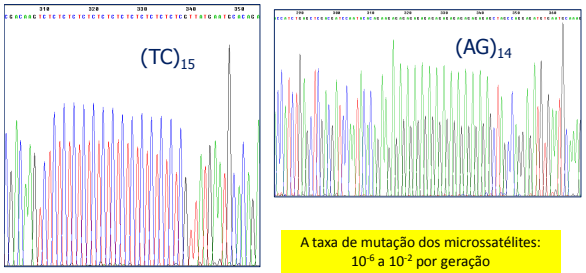
$(CA)_n\ (AG)_n\ (AT)_n$

CACACACACACACACACACACACACACACACAC

Dinucleotideos

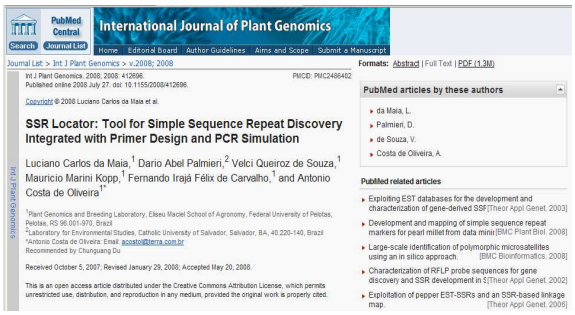


Microsatélites



A taxa de mutação dos microssatélites: 10^{-6} a 10^{-2} por geração

Microsatelites



Minisatelites

atcgtactactagat**TTAGGG**TTAGGG**TTAGGG**TTAGGG**TTAGGG**ttagggatcgctagc

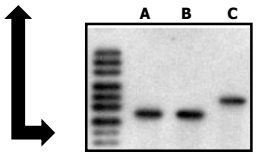
Primer 1 → **Hexanucleotídeos**

A **TTAGGG**TTAGGG**TTAGGG**TTAGGG

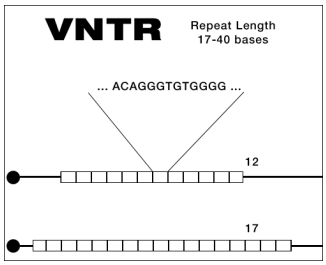
B **TTAGGG**TTAGGG**TTAGGG**TTAGGG

C **TTAGGG**TTAGGG**TTAGGG**TTAGGG**TTAGGG**TTAGGG

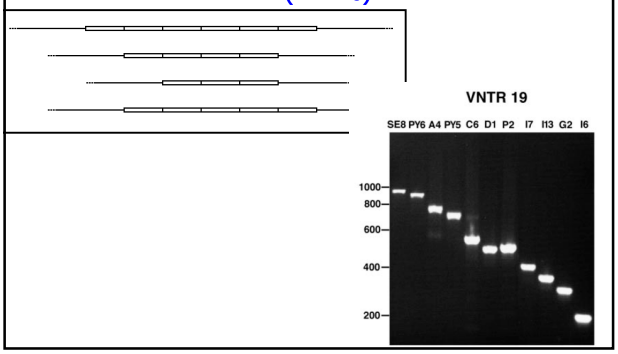
Primer 2 ←



Variable Number of Tandem Repeats

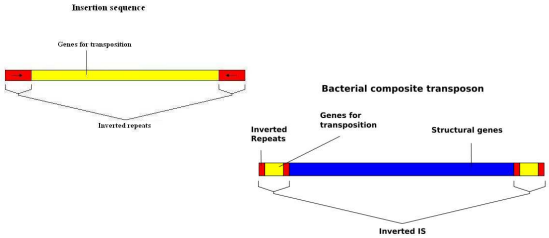


Variable Number of Tandem Repeats (VNTRs)



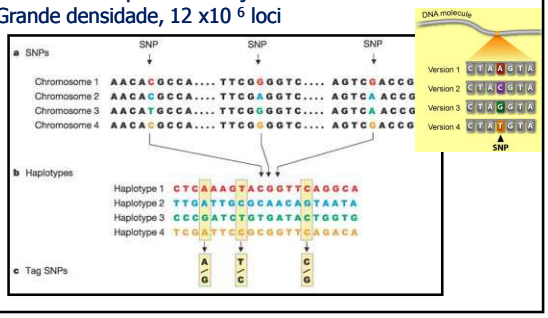
Sequencias de Inserção

- São sequencias pequenas, em torno de 1Kb
- Possuem genes para transposição nos genomas



SNP

- Polimorfismos por substituição de um nucleotídeo
- Grande densidade, 12 x10 ^{6} loci



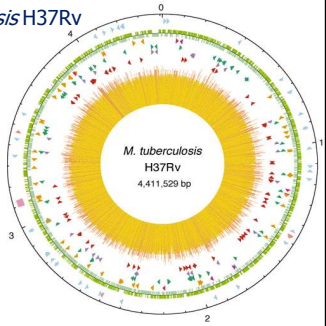
Bactérias

- Regiões PGRS
- DNA repetitivo- SSR,IS, VNTR
- 16S-ITS
- MLST

Mycobacterium tuberculosis

- *Mycobacterium tuberculosis* H37Rv
- Sequenciada em 1998
- Regiões PGRS
- DNA repetitivo –IS (56)
- SNPs

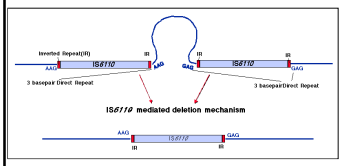
- **IS6110-RFLP**
- **Spoligotyping**
- **MIRU-VNTR**

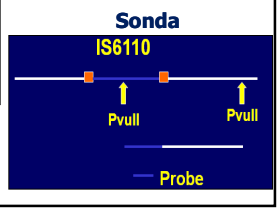


Mycobacterium tuberculosis

•IS6110-RFLP
Restriction Fragment Length Polymorphism

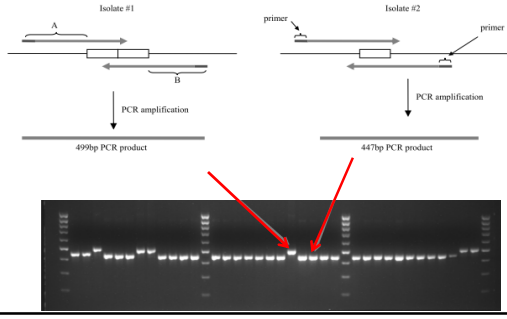
• Estão presentes em posição e numero variável no genoma





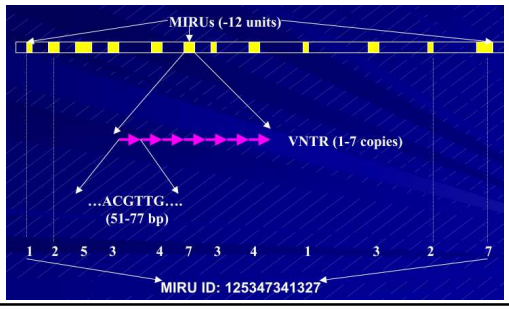
Mycobacterium tuberculosis

MIRU-VNTR
Mycobacterial Interspersed Repetitive-Unit-Variable-Number Tandem-Repeat



Mycobacterium tuberculosis

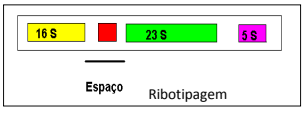
MIRU-VNTR
12 Unidades Repetidas Independentes



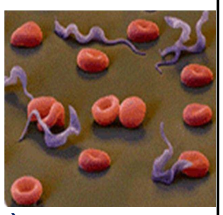
Streptococcus pneumoniae

•Um dos agentes causadores da meningite bacteriana

- •90 sorotipos (**14**,**6**,18,**19**,**23**,49,7,1 e 3)
- •Genoma com 2.1Mb
- •Alvos 16S-23S-ITS, ply, lyt, psaA, pbp2b, pbp1A
- •5 % do genoma com IS
- •SNPs



Trypanossoma cruzi



- Genoma entre 106,4 e 110,7 Mb
- Diplóide – 41 pares de cromossomos
- 50% do genoma DNA repetitivo
  - •Retrotransposons
  - •Proteínas de superfície (MASP, gp63...)
- DNA do cinetoplasto (kDNA)
- VNTR

Trypanossoma cruzi

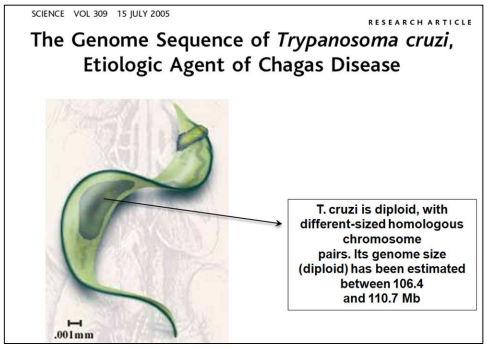
Marcadores Moleculares

- Região 3' do domínio 24Sα do gene do rRNA
- Gene que codifica para a subunidade 2 da oxidase citocromo mitocondrial (COII)

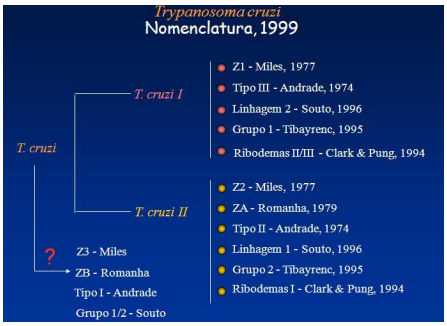
•Microssatelites (SCLE10, SCLE11, MCLF10-locus de repetição CA(n) e TcTAT20, TcAAT8, TcAAAT6)

A.C.J., et al. Acta Trop. (2010)

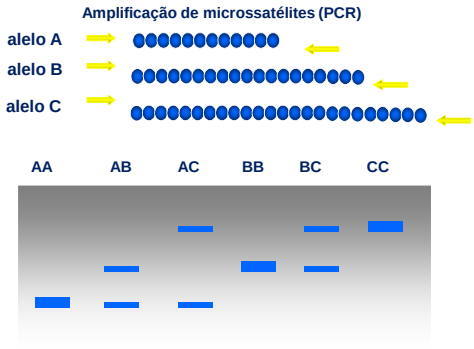
Trypanossoma cruzi



Trypanossoma cruzi



Trypanossoma cruzi



Trypanossoma cruzi

24Sα do gene do rRNA

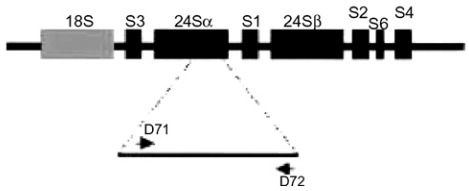


Fig. 2: The ITS 1 separates the coding region of the 18S subunit and the 5.8 S rDNA, and the ITS2 separates 5.8 S rDNA sequences from the 24S rDNA. rDNA typing is performed using primers D71 and D72 that amplifies a dimorphic region at the 3' end of the 24Sa rRNA gene.

Trypanossoma cruzi

Isoenzimas

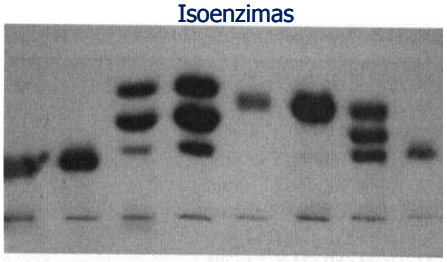


Fig. 15.6 Isoenzyme profiles of *Trypanosoma cruzi* isolates by starch-gel electrophoresis.

Toxoplasma gondii

- Possui genoma de 78 Mb
- 11 cromossomos
- Famílias de elementos repetitivos
  - ABGTg ou TGR
  - TgIRE
- rRNA
- SAG
- GRA
- SNPs

Toxoplasma gondii

PCR-RFLP

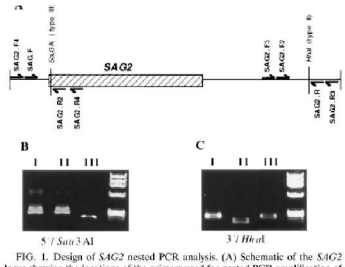
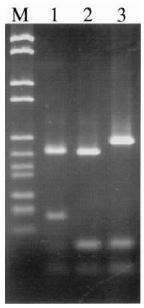


FIG. 1. Design of *SAG2* nested PCR analysis. (A) Schematic of the *SAG2* locus showing the locations of the primers used for nested PCR amplification of the 5′ and 3′ ends of the locus and the polymorphic restriction sites used for identification of strain genotypes. The hatched box represents the open reading frame of the *SAG2* gene. (B) *Sau*3AI restriction analysis of the 5′ amplification products from type I, II, and III strains. (C) *Hha*I restriction analysis of the 3′ amplification products from type I, II, and III strains. Products were resolved in 1.2% agarose gels stained with ethidium bromide. Molecular weight markers correspond to ϕX174 digested with *Hae*III.

SAG2 locus SAG2- encoding tachyzoite surface antigen p22

Howe et al.J Clin Microbiol, 1997, p. 1411±1414

Toxoplasma gondii



PCR-RFLP

GRA6

Fig. 1. PCR-RFLP analysis of *GRA6* gene coding region with *Mse*I endonuclease. Lane M is DNA size marker VI (between 2176 and 174 bp). Lanes 1–3 are *Toxoplasma gondii*, type I (RH), type III

A. Fazaeli et al. / International Journal for Parasitology 30 (2000) 637±642

Toxoplasma gondii

PCR-RFLP e sequenciamento
(5'SAG2, 30-SAG2, SAG3 and GRA6)

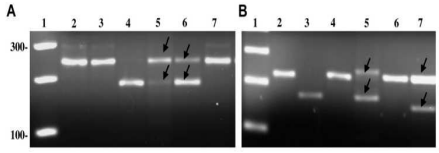
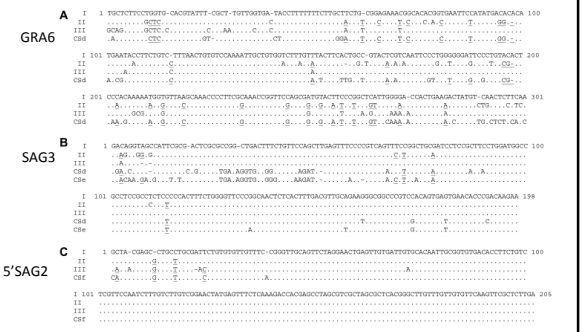


Fig. 1. Restriction patterns of PCR products amplified from clinical samples with recombinant *T. gondii* strains. 5′-SAG2 (A) and 3′-SAG2 (B) amplification products were digested with *Sau*3AI and *Hha*I, respectively. The DNA fragments were resolved in 2% agarose gels stained with ethidium bromide. Lane 1, 100-bp ladder (the lowest band shown is 100 bp); lane 2, RH (type I); lane 3, ME49 (type II); line 4, VEG (type III); lines 5–7, CSa, CSb and CSc (clinical samples). The arrows show the allele combinations.

I.M.R. Ferreira et al. / Exper Paras. 118 (2008) 221–227

Toxoplasma gondii

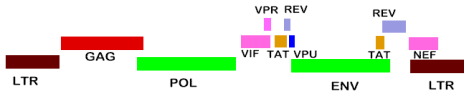
PCR-RFLP e sequenciamento



I.M.R. Ferreira et al. / Exper Paras. 118 (2008) 221–227

Marcadores Moleculares

Ex: O genoma do HIV



HIV-1 GENOME 9749 NUCLEOTIDES

- Três genes estruturais
- Sequencias repetitivas regulatórias (LTRs)
- Genes regulatórios (não empacotados no virion)

Caracterização Molecular

191 Kpb / 263 genes

ITRs | Genes Conservados | ITRs

Variáveis - Genes não-essenciais – interação com o hospedeiro e virulência

Genes essenciais – polipeptídeos estruturais e enzimas envolvidas no metabolismo da DNA

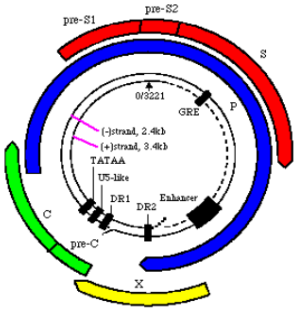
Caracterização Molecular

Alvos

- Genes Estruturais
- Regiões LTR (Long Terminal Repeat)
- ITRs
- SNPs

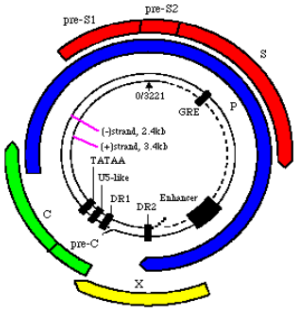
Vírus Hepatite B

- Genoma de 3.2kb
- 4 genes (C, S, P, X)
- 9 Sorotipos: *awy1, awy2, awy3, awy4, ayr, adw2, adw4, adrq-, adrq+*
- 8 genótipos (A-H) No Brasil : A, D e F



Vírus Hepatite B

- Alvos - Genes C e S
  - SNPs



Vírus Hepatite B

- Determinação de mutações
- S e C
- PCR e Digestão AvaII and MspI

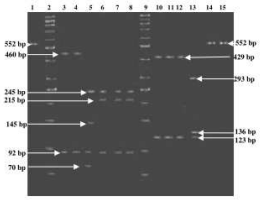
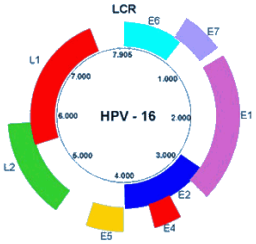


Fig. 2. Gel image of RFLP assay for detection of HBV variants for core region after Ava II and Msp I enzyme digestion. *1* Uncut DNA fragment (552 bp); *2*, *9* A 50-bp ladder (Fermentas); *3*, *4* A1 mutants (460, 92 bp); *5* A2 mutants (245, 145, 70, 92 bp); *6–8* A4 mutants (245, 215, 92 bp); *10–12* M1 mutant (429, 123 bp); *13* M2 mutant (136, 293, 123 bp); *14*, *15* M3 mutant (552 bp)

M. A. Aslam et al. Arch Virol (2008) 153: 163–170

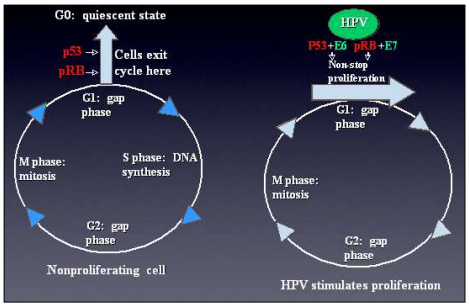
Papilomavírus Humano

- Família Papillomaviridae
- DNA fita dupla circular
- Genoma 7.9Kb
- Mais de 120 tipos
- Tipo 16 presente em 60-70% dos carcinomas
- **Proteínas E6 ou E7**
- LR (6, 11, 42, 43, 44)
- HR (16, 18....)



Papilomavírus Humano

•Proteínas E6 ou E7



Papilomavírus Humano

PCR-RFLP
Alvo E7

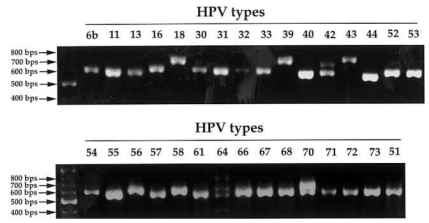
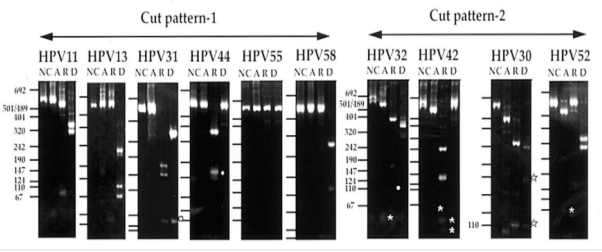


Fig. 1. Detection of mucosal HPV types with LCR-W PCR. Four picograms of cloned HPV DNA (all types except for HPV51) and 100 ng of cellular DNA from a clinical sample which was infected with HPV51 were used for amplification with PCR. Three microliters; of PCR products were run on agarose gels and viewed with a UV-monitor camera after ethidium bromide staining.

T. Sasagawa et al. : Virus Research 67 (2000) 127±139

Papilomavírus Humano

PCR-RFLP



T. Sasagawa et al. : Virus Research 67 (2000) 127±139

Papilomavírus Humano

PCR-RFLP

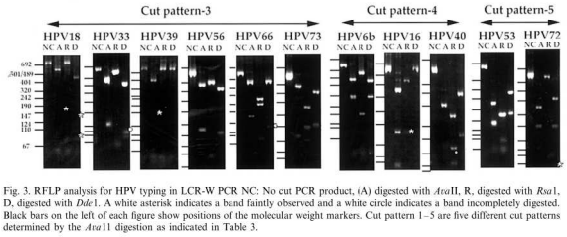


Fig. 3. RFLP analysis for HPV typing in LCR-W PCR NC: No cut PCR product, (A) digested with *Ara*II, R, digested with *Rsa*1, D, digested with *Dde*1. A white asterisk indicates a band faintly observed and a white circle indicates a band incompletely digested. Black bars on the left of each figure show positions of the molecular weight markers. Cut pattern 1–5 are five different cut patterns determined by the *Ara*11 digestion as indicated in Table 3.

T. Sasagawa et al. : Virus Research 67 (2000) 127±139

Técnicas moleculares

RFLP - restriction fragment length polymorphism DNA
RAPD - random amplified polymorphic DNA
CAPS - cleveaded amplified fragment sequence
SSR - Simple Sequence Repeat / Microssatélites
AFLP - amplifided fragment length polymorphismic DNA
SNP - single nucleotide polymorphisms

As diferentes classes de marcadores moleculares podem diferir com respeito a características importantes como:
abundância genômica
nível de polimorfismo detectado
especificidade dos locos
reprodutibilidade
labor

Marcadores Moleculares

- RFLP
- VNTR (minissatélite)
- RAPD
- PCR-específico - SSR, ISSR, CAPS
- MLST
- AFLP
- SNPs

Marcadores Moleculares- Histórico

RFLP proposto por Botstein et *al.* (1980)
descrito para humanos

PCR proposto por Mullis & Faloona (1987)

VNTR por Jeffrey (1987)

RAPD por Rafalski *et al.* (1990)

Marcadores Moleculares- Histórico

SSR em plantas por Akkaya *et al.* (1992)

AFLP por Zabeau & Vos (1993)

CAPS por Konieczny & Ausubel (1993)

SNPs em Arabidopsis po Cho *et al.* (1999) -

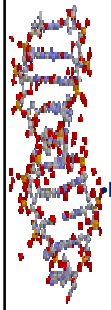
Marcadores Moleculares- Tipo de Técnica

- Métodos sem uso de PCR
  - RFLP
- Métodos com uso de PCR
  - PCR com primers arbitrários
    - RAPD, AP-PCR, DAF (DNA amplification fingerprint),
    - AFLP;
    - ISSR
  - PCR sítio-específico
    - CAPS, SCAR
    - SSRs (microssatélites)

Marcadores Moleculares- N° de Cópias

- Seqüência de poucas cópias - codificante
  - RFLP
- Seqüência com cópias repetidas
  - VNTR
  - SSRs (microssatélites)
  - ISSR
- Seqüência com número de cópias indefinido
  - RAPD, AP-PCR, DAF
  - AFLP
  - CAPS, SCAR

Técnicas de Caracterização Molecular



•Baseados em PCR

•Baseado em Hibridização de Ácidos Nucléicos

•Baseado no sequenciamento de Ácidos Nucléicos

Caracterização Molecular baseada em PCR

RAPDs (Randomly Amplified Polymorphic DNA)
ALFP (Amplifid Fragment Length Polymorphisms)
SSR-PCR (Short Sequence Repeats PCR)
PCR-RFLP
MLST- Multi Locus Sequence Typing

RAPDs (Randomly Amplified Polymorphic DNA)

•Utiliza primers pequenos (10bp) para amplificar randomicamente fragmentos de DNA

•Baixa Tm

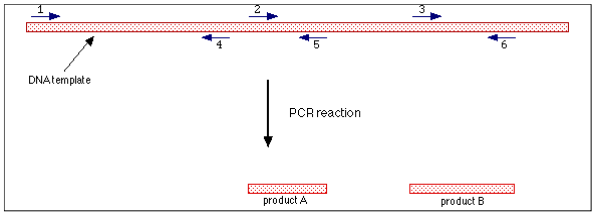
•Na ha a necessidade de informações sobre o genoma a ser amplificado

• Requer pelo menos 10 primers

5´GGTGCGGGAA 3´
5' GGTGCGGGAA 3´
5´GTTTCGCTCC 3´
5´GTAGACCCGT 3´
5´AAGAGCCCGT 3´

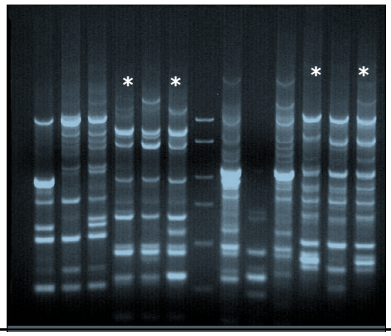
RAPDs (Randomly Amplified Polymorphic DNA)

Princípio



RAPDs (Randomly Amplified Polymorphic DNA)

Para cada banda- escore = **presença/ ausência da banda**



RAPDs (Randomly Amplified Polymorphic DNA)

- Marcadores RAPD são anônimos
- Dados binários (presença x ausência)
- Problemas de co-migração
  - mesma banda, mesmo fragmento?
  - uma banda, um fragmento?

AFLP (Amplifid Fragment Length Polymorphisms)

Procedimento dividido em 4 etapas:

•1- Digestão do DNA total com enzimas de restrição

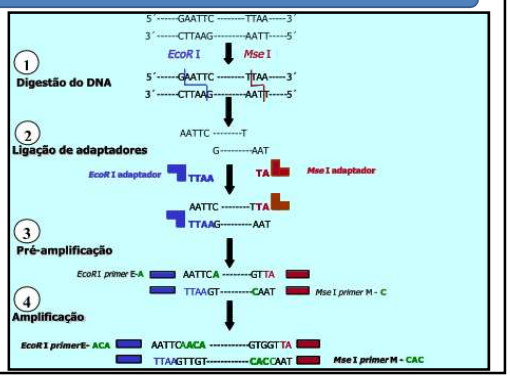
•2-Ligação de adaptadores sitio-especificos

•3- Pré-PCR de alguns fragmentos

•4- PCR

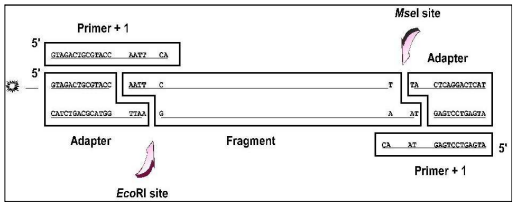
AFLP (Amplifid Fragment Length Polymorphisms)

Princípio



Marcadores Moleculares

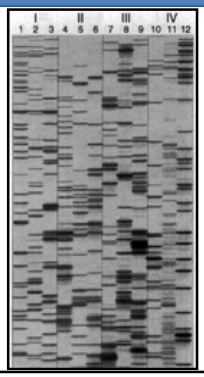
AFLP (Amplifid Fragment Length Polymorphisms)



Marcadores Moleculares

AFLP (Amplifid Fragment Length Polymorphisms)

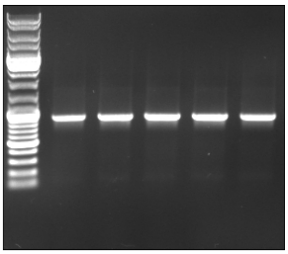
- Eletroforese em gel de poliacrilamida
- Coloração com prata

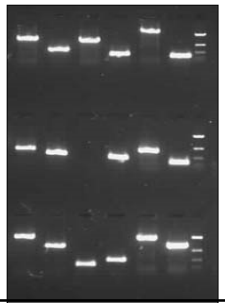


Marcadores Moleculares

PCR-RFLP

PCR – Digestão
CAPS - cleveaded amplified fragment sequence

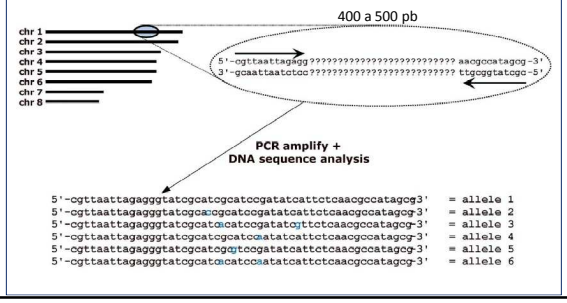




Marcadores Moleculares

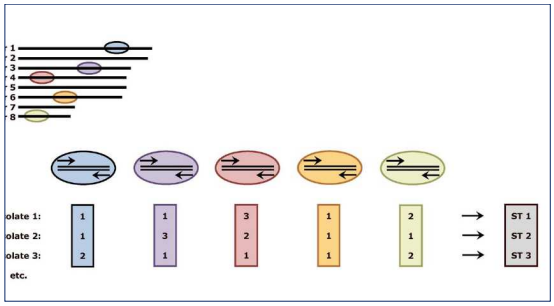
MLST- Multi Locus Sequence Typing

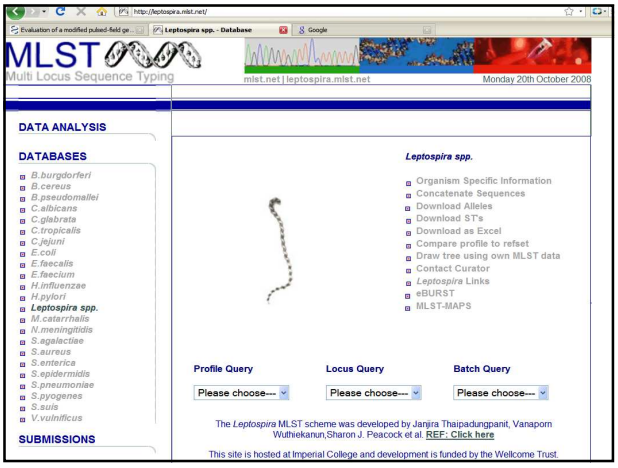
Principle

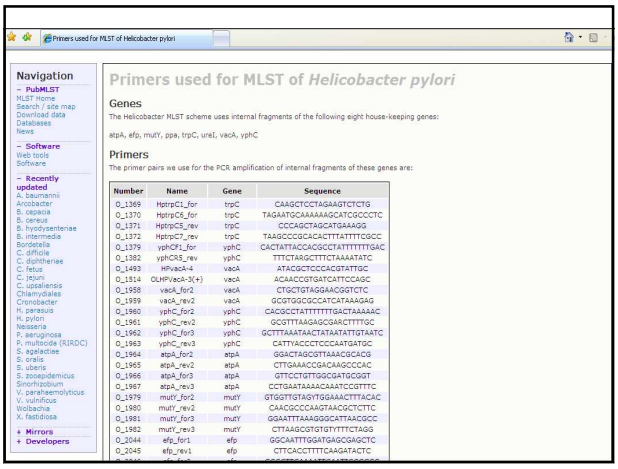


Marcadores Moleculares

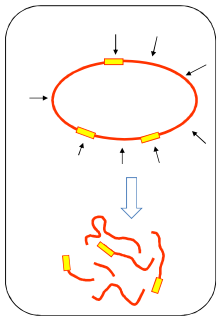
MLST- Multi Locus Sequence Typing







PFGE (Pulsed Field Gel Eletrophoresis)



Eletroforese em Campo Pulsado

- DNA e digerido com enzimas de restrição
- Eletroforese

Equipamento

PFGE (Pulsed Field Gel Eletrophoresis)

Princípio

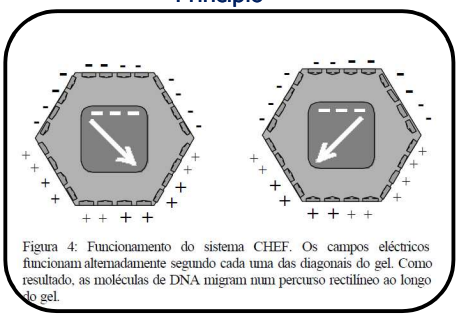
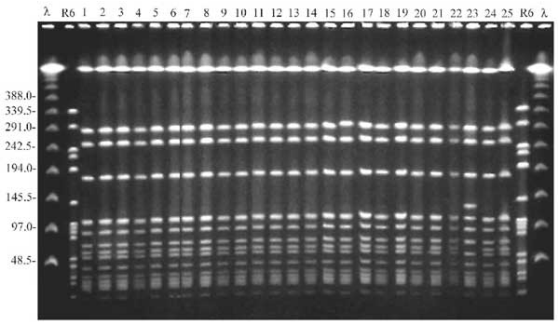


Figura 4: Funcionamento do sistema CHEF. Os campos eléctricos funcionam alternadamente segundo cada uma das diagonais do gel. Como resultado, as moléculas de DNA migram num percurso rectilíneo ao longo do gel.

PFGE (Pulsed Field Gel Eletrophoresis)



Caracterização Molecular baseada em Hibridização

(RFLP) Restriction Fragment Length Polymorphisms

(RH) Hibridização reversa

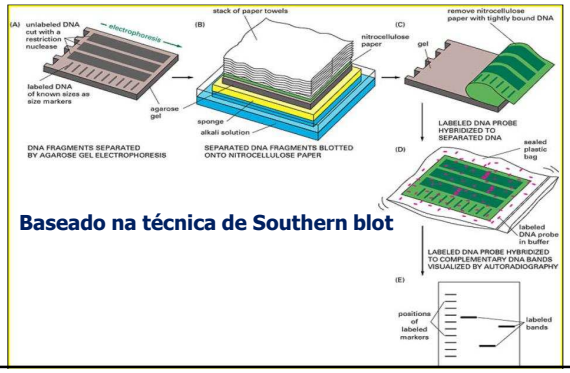
RFLP

Análise de restrição de fragmentos polimórficos

Procedimento dividido em 4 etapas:

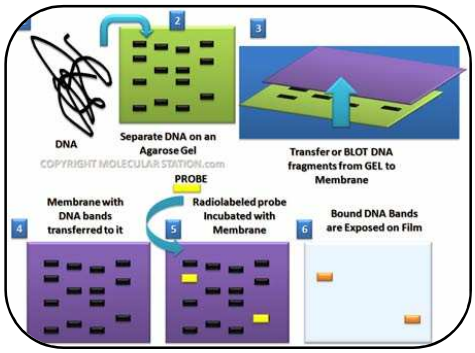
- 1-Digestão com enzimas de restrição
- 2-Eletroforese em gel de agarose
- 3-Transferência para uma membrana de nylon
- 4-Hibridização com sonda de DNA

RFLP

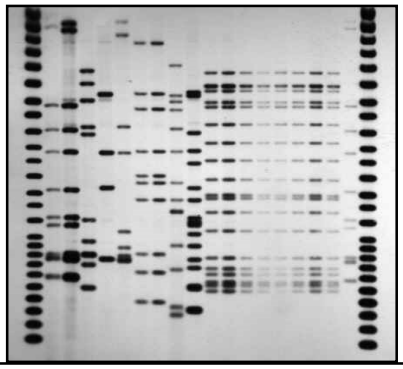


Baseado na técnica de Southern blot

RFLP



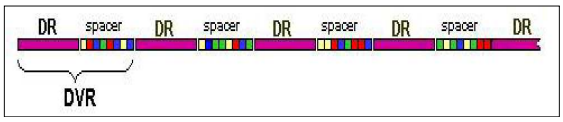
RFLP- Resultado



Hibridização Reversa

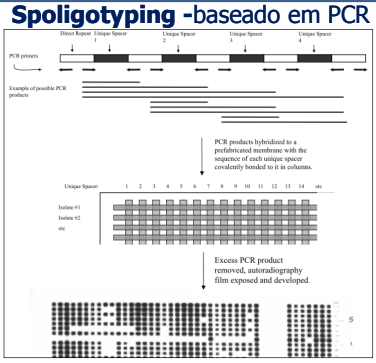
Spoligotyping

PCR- Dra 5'-GGTTTTGGGTCTGACGAC-3'
Drb 5'-CCGAGAGGGGACGGAAAC-3'



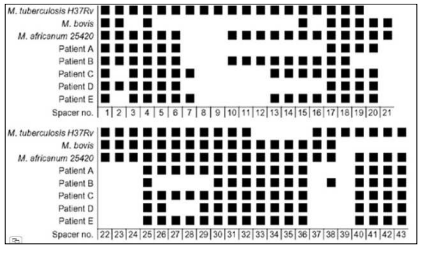
Hibridização Reversa

Spoligotyping -baseado em PCR



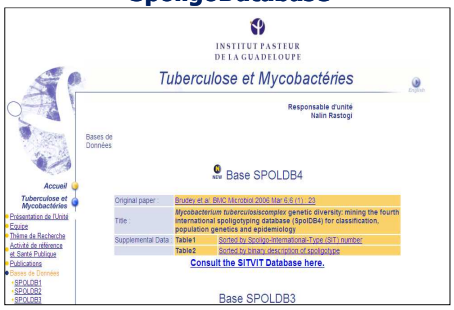
Hibridização Reversa

- Atualmente 94 diferentes espaçadores foram identificados
- 43 espaçadores (35 a 41 pb) são utilizados para a diferenciação de MCT



Mycobacterium tuberculosis

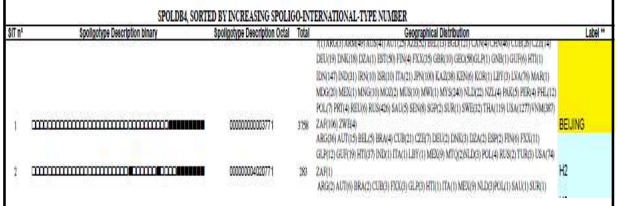
SpoligoDatabase



http://www.pasteur-guadeloupe.fr/tb/bd_myco.html

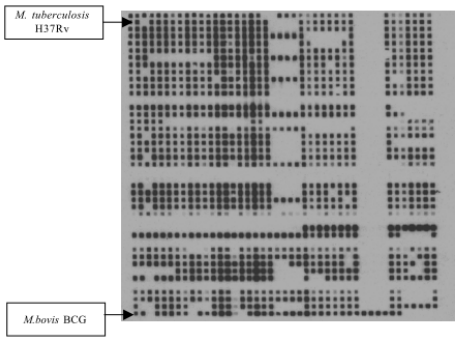
Mycobacterium tuberculosis

SpoligoDatabase



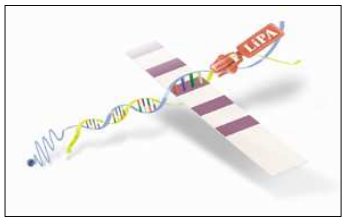
http://www.pasteur-guadeloupe.fr/tb/bd_myco.html

Hibridização Reversa



Hibridização reversa

- Sonda esta numa membrana
- DNA do patógeno e utilizado para hibridizar a sonda



DNA/RNA viral

SNPs

Single Nucleotide Polymorphism

- SNPs são as mais freqüentes formas de variações genéticas

- SNPs tem se tornado marcadores de preferência pela sua grande abundância e pelo desenvolvimento de tecnologias de genotipagem em larga escala.

SNPs

SNP - Estratégias

1) Primer extension (nucleotide incorporation)

2) Clivagem enzimática

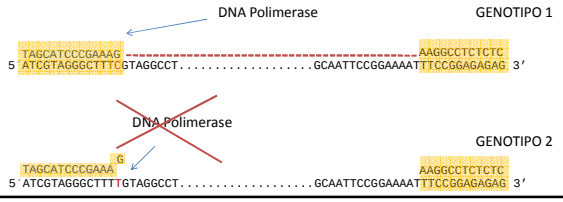
3) Hibridização

SNPs

1) primer extension –PCR

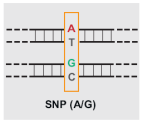
- UM PRIMER DEVE TER SEU ULTIMO NUCLEOTIDEO 3´ SOBRE O NUCLEOTIDEO ALVO

Durante a polimerização de um fragmento (*amplicon*) de DNA pela *DNA Polimerase*, ela necessita de que o ultimo nucleotideo da posição 3´ onde o *primer* esta ligado, tenha uma conformação ABRUPTA

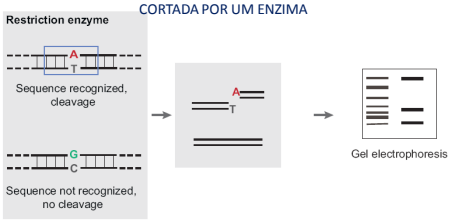


SNPs

2) PCR e Clivagem enzimática



A) CONJUNTO DE PRIMERS QUE AMPLIFICAM UMA REGIAO ALVO
B) A REGIAO ALVO (DENTRO DO AMPLICON) É CORTADA POR UM ENZIMA



SNPs

2) PCR e Clivagem enzimática

SNPs

2) PCR e Clivagem enzimática

GENOTIPO 1

3´TAGCATCCCGAAAGCATCCGGAATAACTTAA-------GCTAATTACGTTAAGGCCTTTTAAAGGCCTCTCTC 5´
5´ATCGTAGGGCTTTCGTAGGCCTTATTG-------AATTCGATTAATGCAATTCCGGAAAATTTCCGGAGAGAG 3´

3´TAGCATCCCGAAAGCATCCGGAATAACTTAA
5´ATCGTAGGGCTTTCGTAGGCCTTATTG

EcoRI
5'...G/AATTC...3'
3'...CTTAA\G...5'

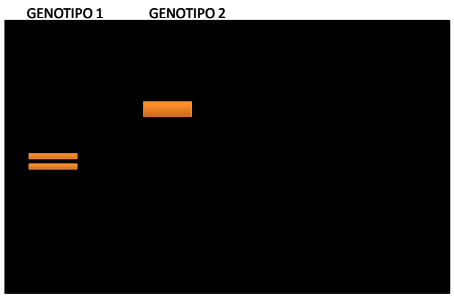
5´ GCTAATTACGTTAAGGCCTTTTAAAGGCCTCTCTC 5´
5´ AATTCGATTAATGCAATTCCGGAAAATTTCCGGAGAGAG 3´

GENOTIPO 2

3´TAGCATCCCGAAAGCATCCGGAATAAATTAAGCTAATTACGTTAAGGCCTTTTAAAGGCCTCTCTC 5´
5´ATCGTAGGGCTTTCGTAGGCCTTATTTAATTCGATTAATGCAATTCCGGAAAATTTCCGGAGAGAG 3´

SNPs

2) PCR e Clivagem enzimática



SNPs

3) Hibridização

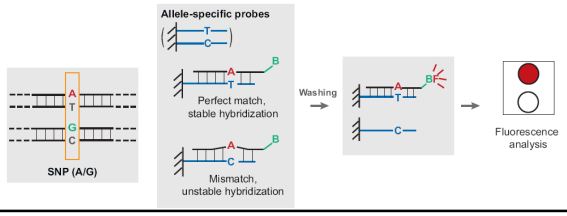
a) Vários locos e vários genes
EX: Microarray de 25-mers (genes)
Microarray Genoma "painel de SNPs"

b) Poucos locos, poucos genes ou gene especifico
EX: sondas *TAQ MAN*

SNPs

3) Hibridização

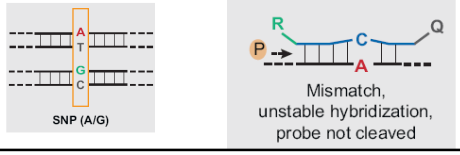
a) Vários locos e vários genes
EX: Microarray de 25-mers (genes) e Microarray Genoma "painel de SNPs"



SNPs

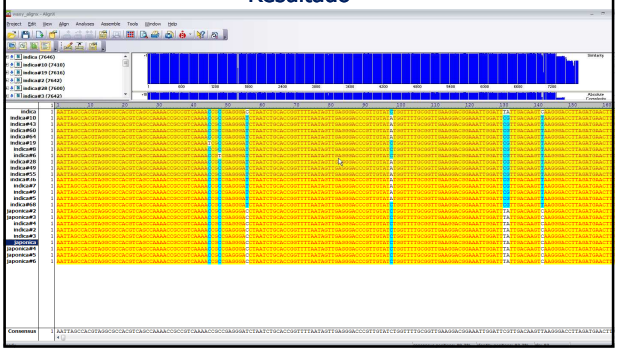
3) Hibridização

b) Poucos locos, poucos genes ou gene especifico
EX: sondas TAQ MAN

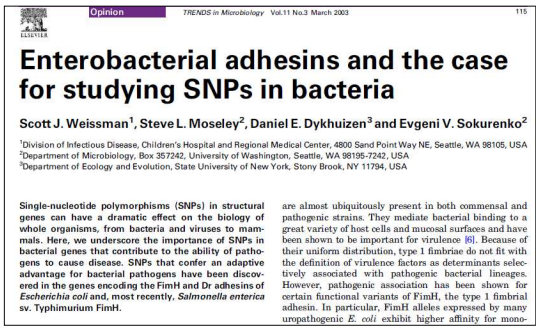


SNPs

Resultado



SNPs



Opinion TRENDS in Microbiology Vol.11 No.3 March 2003 115

Enterobacterial adhesins and the case for studying SNPs in bacteria

Scott J. Weissman[1], Steve L. Moseley[2], Daniel E. Dykhuizen[3] and Evgeni V. Sokurenko[2]

[1]Division of Infectious Disease, Children's Hospital and Regional Medical Center, 4800 Sand Point Way NE, Seattle, WA 98105, USA
[2]Department of Microbiology, Box 357242, University of Washington, Seattle, WA 98195-7242, USA
[3]Department of Ecology and Evolution, State University of New York, Stony Brook, NY 11794, USA

Single-nucleotide polymorphisms (SNPs) in structural genes can have a dramatic effect on the biology of whole organisms, from bacteria and viruses to mammals. Here, we underscore the importance of SNPs in bacterial genes that contribute to the ability of pathogens to cause disease. SNPs that confer an adaptive advantage for bacterial pathogens have been discovered in the genes encoding the FimH and Dr adhesins of *Escherichia coli* and, most recently, *Salmonella enterica* sv. Typhimurium FimH.

are almost ubiquitously present in both commensal and pathogenic strains. They mediate bacterial binding to a great variety of host cells and mucosal surfaces and have been shown to be important for virulence [6]. Because of their uniform distribution, type 1 fimbriae do not fit with the definition of virulence factors as determinants selectively associated with pathogenic bacterial lineages. However, pathogenic association has been shown for certain functional variants of FimH, the type 1 fimbrial adhesin. In particular, FimH alleles expressed by many uropathogenic *E. coli* exhibit higher affinity for mono-

Analises dos Resultados

- Analise dos dados obtidos pela caracterização molecular
- Agrupamento dos perfis moleculares similares- Dendograma
- Analises filogenéticas

Utilização

Caracterização Molecular

- Conhecer a distribuição de sorotipos e a diversidade genética
- Conhecer a distribuição de cepas Resistentes aos antimicrobianos
- Identificação de surtos
- Determinar a dinâmica da transmissão em áreas geográficas
- Identificar fatores de risco para a ocorrência das doenças infecciosas

Utilização

Caracterização Molecular

- Identificar determinantes de patogênese
- Rotas de transmissão
- Reatividade sorológica
- Resposta a terapias anti-virais, anti-bacterinas ou anti-parasitarias
- Utilizar os dados em Estudos epidemiológicos