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POLIMORFISMO NO GENE DA MBL-2 EM MULHERES INFECTADAS POR *Chlamydia trachomatis*, COFATOR DO CÂNCER CERVICAL

Recife-PE
2008

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POLIMORFISMO NO GENE DA MBL-2 EM MULHERES INFECTADAS POR *Chlamydia trachomatis*, COFATOR DO CÂNCER CERVICAL

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"A vida sem ciência é uma espécie de morte"

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"Os dias prósperos não vêm por acaso; nascem de muita fadiga e muita persistência"

Henry Ford.

RESUMO

A *Chamydia trachomatis (C. trachomatis)* é uma bactéria intracelular obrigatória, sexualmente transmitida, que causa doença inflamatória pélvica, gravidez ectópica e infertilidade. O papilomavírus humano (HPV) e a *C. trachomatis* são responsáveis por infecções sexualmente transmissíveis (ISTs) tipo-específicas, que infectam a pele, mucosas e o trato genital masculino e feminino, causando lesões, tanto benignas quanto malignas. A lectina ligadora de manose (MBL, *Mannose-Binding Lectin)* é uma proteína sérica da resposta imune inata capaz de ativar o sistema complemento e de se ligar a resíduos de carboidratos, inclusive a manose, em diferentes microrganismos. A deficiência ou polimorfismo gênico, dessa proteína, associa-se a um aumento da susceptibilidade a muitas doenças infecciosas. Os objetivos principais desse trabalho foram elaborar um protocolo para detecção da infecção por *C. trachomatis* e investigar a freqüência do polimorfismo no primeiro éxon do gene MBL2, correlacionando-os com a susceptibilidade à infecção por *C. trachomatis,* através da reação da PCR em Tempo Real (Real-Time *Polimerase Chain Reaction*), utilizando o SYBR® Green como fluoróforo. Para determinação do protocolo de identificação da *C. Trachomatis* foram utilizadas 98 amostras de secreção vaginal, oriundas de pacientes do Ambulatório Especializado da Mulher, da Prefeitura Municipal do Recife-PE, Brasil. O diagnóstico da infecção foi dado pela análise *melting temperature assay.* Aproximadamente, 14% (n=14) das amostras foram positivas para a infecção. Todas as amostras foram confirmadas através da análise em gel de agarose a 1%. Em relação à associação do polimorfismo do gene MBL2 entre os grupos infectados e saudáveis, os resultados demonstraram que não houve diferença estatística na freqüência dos alelos entre os grupos (64% *vs.* 74%) (36% vs. 26%), respectivamente, alelos A e 0, *p-value* = 0.3112), não sendo associado a um aumento na susceptibilidade à infecção pela *C. trachomatis*. Da mesma forma, quando comparadas às freqüências genotípicas, o genótipo "OO", no grupo *C. trachomatis*-positivo, não mostrou diferença significativa em relação ao grupo controle, 6% *vs.* 3%, respectivamente. Além disso, apesar do genótipo "AA", no grupo controle, apresentar uma freqüência maior em relação ao grupo *C. trachomatis*-positivo (47% vs. 33%), esta não foi significativa (*p-value* = 0.1430). Todas as amostras estavam em equilíbrio de *Hardy-Weinberg* (χ^2=2,65). Conclui-se assim que com a metodologia proposta, pode-se facilmente detectar a presença de *C. trachomatis,* em amostras de secreção vaginal, e que a presença do alelo mutante "O" no gene da MBL2, não confere fator predisponente relevante para um aumento na susceptibilidade à infecção por *C. trachomatis.*

Palavras-chave: *Chlamydia trachomatis;* MBL-2; PCR.

ABSTRACT

Chlamydia trachomatis (C. trachomatis) are obligate intracellular bacteria transmitted by sexual contact that provoke pelvic inflammations, ectopic pregnancy and infertility. Papilomavírus human (HPV) and the *C. trachomatis* is responsible for sexually transmissible infections (ISTs) type-specific, that the skin, mucosae and the masculine and feminine genital treatment infectam, causing injuries, benign how much in such a way malignant. Mannose-Binding Lectin (MBL) is an important molecule of immune system due to its ability to activate the complement pathway. The deficiency or genic polymorphism of this protein, associates it an increase of the susceptibility to many infectious illnesses. The main purposes of this research had been to elaborate a protocol for detection of the infection for *C. trachomatis* and to investigate the frequency of the polymorphism in the first of MBL2 gene in patients being correlated with susceptibility to the infection for *C. trachomatis* infection, through the reaction of the Real-Time PCR (Real-Time Polimerase Chain Reaction) using the SYBR® Green as fluoróforo. For determination of the protocol to identification of *C. trachomatis,* 98 samples of vaginal secretions, from the Women Ambulatory Specialized from Recife-PE, Brazil. The diagnostic of the infection was given by the analysis melting temperature assay using Rotor Gene™ Real Time PCR as platform. Approximately, 14% (n=14) of the samples had been positive for the infection. All the samples had been confirmed through the analysis in 1% agarose gel. In relation of the association of the polymorphism of MBL2 gene between infected and health groups, the results had demonstrated that it did not have difference statistics in the frequency of those allelic ((64% versus. 74%) (36% versus. 26%), respectively alleles A and 0, p-value = 0.3112), not being associated to an increase in the susceptibility to the infection for the *C. trachomatis*. In the same way, when compared with the genotype frequency, the genotype "OO", in group *C. trachomatis*-positive, did not show significant difference in relation to health group, 6% versus. 3%, respectively. Moreover, although genotype "AA", in the health group, to present a bigger frequency in relation to study group (47% versus. 33%) this were not significant (p-value = 0.1430). All the samples were in balance of Hardy-Weinberg (χ^2=2,65). In conclusion, using this metodology proposal we can easily detect the presence of *C. trachomatis* in samples of vaginal secretions and that the presence of mutant allele "0" in the gene of the MBL-2, does not confer excellent predisponent factor for an increase in the susceptibility to the infection for *C. trachomatis.*

Keywords: Chlamydia trachomatis; MBL-2; PCR.

LISTA DE FIGURAS

LISTA DE TABELAS

LISTA DE ABREVIATURAS

Sigla	*Significados*	
	Português	Inglês
Ac	Anticorpo	Antibody
Ag	Antígeno	Antigen
AGUS	Atipias de Células Glandulares de Significado Indeterminado	Atypical Glandular Cells of Undetermined Significance
APC	Célula Apresentadora de Antígeno	Antigen Presented Cell
Arg	Arginina	Arginine
ASCUS	Atipias de Células Escamosas de Caráter Desconhecido	Atypical Squamous Cells of Characters Unknown
ATP	Adenosina-5'-Trifosfato	Adenosine-5'-triphosphate
CD	Célula Dendrítica	Dendritic Cell
CL	Célula de Langerhan	Langerhan Cell
C. pneumoniae	*Chlamydia pneumoniae*	*Chlamydia pneumoniae*
C. psittaci	*Chlamydia psittaci*	*Chlamydia psittaci*
C. trachomatis	*Chlamydia trachomatis*	*Chlamydia trachomatis*
CDK	Quinase Ciclina-Dependente	Dependent-Cyclin Kinase
CYC	Ciclina	Cyclin
DNA	Ácido desoxirribonucléico	Deoxyribonucleic acid
DRC	Domínio de Reconhecimento de Carboidratos	Carbohydrates Recognition Domain
dNTP	Dinucleotídeotrifosfato	Dinucleotidetriphosphate
EB	Corpo Elementar	Elementary Body
EGFR	Receptor do Fator de Crescimento Epidérmico	Epidermal Growth Factor Receptor
ELISA	Ensaio Imunoenzimático Enzima-Conjugado	Enzyme-Linked Immunosorbent Assay
EP	Gravidez Ectópica	Ectopic Pregnancy
FDA	Administração de Drogas e Alimentos	Food and Drug Administration
GM-CSF	Fator Estimulador de Colônias	Granulocyte-macrophage

	de Macrófago-Granulócito	colony-stimulating factor
HISL	Lesão Intraepitelial Escamosa de Alto Risco	High-grade Squamous Intraepithelial Lesion
HIV	Vírus da Imunodeficiência Humana	Human Immunodeficiency Virus
HPV	Papilomavírus Humano	Human Papillomavirus
HSV	Vírus Herpes Simples	Herpes Simplex Virus
HSP	Proteína de Choque	Heat Shock Protein
HTLV	Vírus T-Linfotrópico Humano	Human T Lymphotropic Virus
IARC	Agência Internacional para pesquisa em Câncer	International Agency For Research On Cancer
IFD	Imunofluorescência Direta	Direct Immunofluorescence
IFN	Interferon	Interferon
IL	Interleucina	Interleucin
INCA	Instituto Nacional de Câncer	National Institute of Cancer
IST	Infecção Sexualmente Transmissível	Sexually Transmitted Infections
LAG	Lesões Intraepiteliais de Alto Grau	High Grade Intraepiteliais Lesions
LBG	Lesões Intraepiteliais de Baixo Grau	Low Grade Intraepiteliais Lesions
LCR	Região Longa de Controle	Long Control Region
LSIL	Lesão Intraepitelial Escamosa de Baixo Risco	Low-grade Squamous Intraepithelial Lesion
LTC	Linfócito T Citotóxico	Cytotoxic T Lymphocyte
LTH	Linfócito T Auxiliar	Helper T Lymphocyte
OMP	Proteína de Superfície de Membrana	Outer-Membrane Protein
OMS	Organização Mundial da Saúde	World Health Organization
MASP	Serino protease associada à MBL	MBL-Associated Serine Proteases
MBL	Proteína Ligadora de Manose	Mannose Binding-Lectin

MHC	Complexo de Histocompatibilidade Principal	Major Histocompatibility Complex
MOMP	Proteína Principal de Superfície de Membrana	Major Outer-Membrane Protein
NIC	Neoplasia Intraepitelial Cervical	Cervical Intraepithelial Neoplasia
NOD	Domínio de Oligomerização Vinculado ao Nucleotídeo	Nucleotide-Binding Oligomerization Domain
NK	Matadora Natural	Natural Killer
OMS	Organização Mundial de Saúde	World Health Organization
ORF	Códigos Abertos de Leitura	Open reading frame
PAMP	Padrão Molecular Associado ao Patógeno	Pathogen-associated Molecular Pattern
PCR	Reação em Cadeia Polimerase	Polimerase Chain Reaction
PCR-RT	PCR em Tempo-Real	Real-Time PCR
PID	Doença Pélvica Inflamatória	Pelvic Inflammatory Disease
Pro	Prolina	Proline
PRR	Receptor de Reconhecimento de Padrão	Pattern Recognition Receptor
PV	Papilomaviroses	Papillomaviruses
RB	Corpo Reticular	Reticular Body
RN	Recém-Nascido	Newborn
RNA	Àcido Ribonucléico	Ribonucleic Acid
SI	Sistema Imunológico	Immune System
SNP	Polimorfismo de um Único Nucleotídeo	Single Nucleotide Polymorphism
SP	Proteína Surfactante	Surfactant Protein
TNF	Fator de Necrose Tumoral	Tumor Necrosis Factor
TLR	Receptor “Toll”	Toll-Like Receptor
URR	Região “super” regulada	"Upper" regulatory region
VLP	Partículas Vírus-Semelhantes	Vírus Like-Particles
WHO	Organização Mundial de Saúde	World Health Organization

SUMÁRIO

1. INTRODUÇÃO GERAL

O papilomavírus humano (HPV) e a *Chlamydia trachomatis (C. trachomatis)* são responsáveis por infecções sexualmente transmissíveis (ISTs) tipo-específicas, que infectam a pele, mucosas e o trato genital masculino e feminino, causando lesões, tanto benignas quanto malignas. Essas ISTs geralmente acometem a população sexualmente ativa, entre 15 e 49 anos de idade (PEREZ, 2001).

Apesar da *C. trachomatis* estar entre os patógenos mais freqüentes para o ser humano, sua infecção se relaciona mais comumente com uma variedade de processos patológicos, tais como: tracoma, uretrite, pneumonia, linfogranuloma venéreo, psitacose e até mesmo aterosclerose, embora a associação do microrganismo como causa deste último processo ainda não está bem confirmada (TRABULSI & ALTERTHUM, 2005). Atualmente, muitos estudos demonstram que a *C. trachomatis* é um cofator para o desenvolvimento de Neoplasia Intraepitelial Cervical (NIC) e outras alterações celulares significativas em mulheres com histórico de infecção pregressa pelo HPV (TAMIM *et al*., 2002; SMITH *et al*., 2002; FINAN *et al*., 2002; SCHIFFMAN & KJAER, 2003).

O interesse na detecção e identificação das diversas subespécies da *C. trachomatis* na infecção genital e nas lesões precoces, em nível de colo uterino, é relevante na busca de uma perspectiva mais ampla desde a prevenção primária até o desenvolvimento de metodologias curativas capazes de minimizar ou erradicar os processos infecciosos. Essas abordagens permitiram a implantação de novas estratégias de saúde que orientem para uma maior avaliação e mudança no

comportamento sexual dos pacientes selecionados, podendo ser úteis nos processos diagnósticos das infecções causadas por essa bactéria (QURESHI & RUDELLI, 2003).

A cultura da *C. trachomatis* é considerada como o método padrão-ouro *(gold-standard)*, enquanto os métodos de não-cultura incluem: citologia para a detecção de inclusões intracitoplasmáticas, enzimaimunoensaio (ELISA, *Enzyme-Linked Immunosorbent Assay*), imunofluorescência direta (IFD), técnicas e hibridização do DNA e reação em cadeia da polimerase (PCR, *Polimerase Chain Reaction*) (MANAVI, 2006; ZHENG & JIANG, 2007). A PCR é mais sensível em comparação com as técnicas de cultura celular, pois tem uma alta sensibilidade e especificidade, e também quando comparada a outros testes utilizados para o diagnóstico dessa bactéria, tais como a IFD e ELISA, que podem gerar alguns resultados falso-positivos, não-condizentes com a situação da paciente (PIÉMONT & JAULHAC, 1995; SCHACHTER, 1997).

A PCR tornou-se um dos principais adventos da biologia molecular e permitiu o enorme avanço na área, como no seqüenciamento do genoma humano, na expressão de genes recombinantes, na determinação rápida e eficiente dos testes de paternidade e de doenças genéticas e infecciosas. A PCR em Tempo Real oferece várias vantagens comparadas à PCR convencional, uma vez que, provém uma rápida reação de amplificação, não necessitando de procedimentos pós-PCR e o sistema fechado reduz o risco de contaminação cruzada (SEGAT *el al*., 2006).

O Sistema Imunológico (SI) é responsável pelo reconhecimento de substâncias estranhas, sua eliminação, remoção células mortas e/ou danificadas, e destruição de células mutantes e cancerosas. O padrão de defesa do hospedeiro está baseado em ações compreendidas entre as respostas imunes inata (Ex.: fagócitos, proteínas

solúveis, como: citocinas, do sistema complemento (Ex.: vias clássica, alternativa e da Lectina Ligadora de Manose (MBL, *Mannose Binding-Lectin*) e adaptativa (Ex.: anticorpos e células efetoras citotóxicas) (PEAKMAN & VERGANI, 1999).

A MBL tem um papel complexo e importante em muitas doenças humanas. O polimorfismo no gene MBL-2 está associado com a susceptibilidade a algumas infecções, tais como àquelas ocasionadas por HIV, HTLV e *Neisseria meningitides* (SUMMERFIELD *et al.,* 1997; HIBBERD & SUMIYA, 1999; PONTES & TAMEGAO-LOPES, 2005). Nessas infecções, a MBL pode atuar diretamente destruindo os agentes etiológicos através da ativação do sistema complemento e opsonização dos mesmos (SEGAT, 2007).

As concentrações séricas de MBL podem ser determinadas por polimorfismos de um único nucleotídeo (SNPs), no gene estrutural (éxon 1 do gene MBL-2: AA, AO ou genótipo OO). O alelo selvagem é referido como A, enquanto que o alelo O representa uma variante dos alelos B, C, D juntos. Os indivíduos com o genótipo selvagem AA apresentam uma concentração sérica mais elevada da MBL, enquanto que àqueles com genótipo OO apresentam uma concentração mais reduzida, devido a uma formação multimérica, assim resultando em função prejudicada. Além disso, os níveis séricos basais MBL são modificados pelos SNPs na região promotora do gene MBL-2 (H/L ou X/Y) (GEIJN *et al*., 2008).

Em infecções do trato genital feminino por *C. trachomatis*, estudos recentes têm mostrado que a MBL participa da proteção frente à infecção clamidial e que variação alélica na sua expressão, resulta em uma diminuição nos níveis séricos da MBL, aliada a um elevado risco da propagação dessas infecções (VARDHANA *et al*., 2004).

Este trabalho visa correlacionar o polimorfismo do gene MBL-2 em amostras de secreção vaginal de mulheres infectadas por *C. trachomatis*, através da tecnologia da PCR em Tempo Real (*PCR-RT)*, co-infectadas pelo HPV ou não.

2. REVISÃO DA LITERATURA

2.1 Câncer

Câncer (do latin CANCER - Caranguejo) é uma tradução latina da palavra grega carcinoma (KRVKINOS) que tem o mesmo significado. Esta palavra é utilizada para designar os tumores malignos, desde os povos antigos, e teve sua origem decorrente da semelhança com o referido crustáceo, pelo seu caráter infiltrativo. Galeno utilizou-a pela primeira vez, aproximadamente 138-201 a.C., para indicar um tumor maligno da mama em que as veias superficiais desses órgãos apareciam intumescidas, assemelhando-se à pata de caranguejo (MURRAD & KATZ, 1996).

Segundo Hersoug & Arnau (2006) existem quatro teorias sobre a gênese do câncer que têm sido discutidas, consideravelmente, tais como: (1) “Dogma Padrão”. O câncer é desencadeado por um conjunto de mutações, em genes específicos, codificadores de supressores de tumor e oncogenes; (2) ”O Dogma Modificado”. A inativação de um ou mais genes envolvidos no reparo de DNA é necessária; (3) “Teoria da Instabilidade Precoce”. Silenciar um ou mais "genes mestres" da divisão celular; (4) “Teoria da Aneuploidia”. Erros na replicação do DNA, durante a divisão celular, resulta em células aneuplóides. As três últimas teorias explicam, sob diferentes maneiras, como a instabilidade genômica pode desencadear o câncer e o desenvolvimento de tumores, pois estas enfatizam o polimorfismo cromossomal, observado na maioria das células tumorais. Os clones resultantes podem rearranjar e expressar genes contribuintes à evasão imune e apoptose.

O câncer é uma doença genética, causada por mutações em genes, denominados de protooncogenes que se transformam em oncogenes. Os protooncogenes normalmente desempenham funções celulares básicas, em geral relacionadas à regulação da multiplicação celular. O oncogene pode surgir não apenas em decorrência da ativação de protooncogenes, promotores do crescimento, mas também devido à inativação de genes que normalmente suprimem a proliferação celular (genes supressores de tumores ou anti-oncogenes). Entretanto, vários tipos de eventos podem transformar um protooncogene em um oncogene, estado no qual ele promove as duas principais características do câncer: (i) multiplicação celular descontrolada, levando a um crescimento excessivo de um grupo de células chamado de tumor e (ii) dispersão de células pelo corpo para formar novos tumores, um processo chamado de metástase (GRIFFITHS *et al.*, 1998; ROBBINS *et al.*, 2001) (Figura 1).

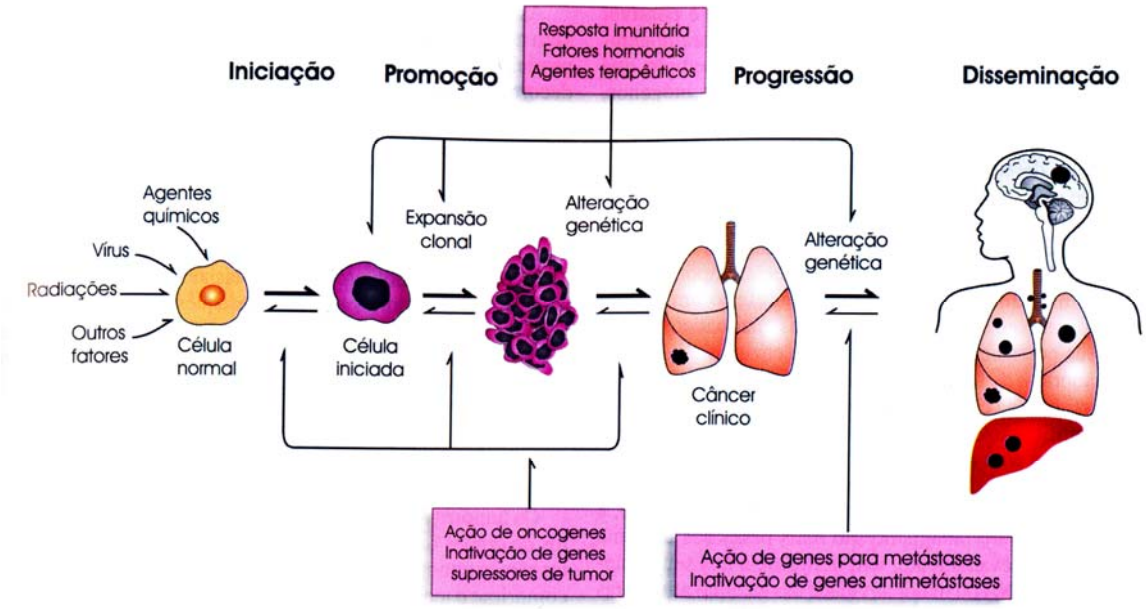

Figura 1. Modelo de carcinogênese em múltiplos estágios apresentando as diferentes fases, onde ocorrem eventos genéticos e epigenéticos com progressão tumoral que se faz em dois sentidos, embora predomine no sentido de maior malignidade. Fonte: (FILHO, 2004).

2.2. Câncer Cervical

O câncer é um importante problema de saúde pública em países desenvolvidos e em desenvolvimento, sendo responsável por mais de seis milhões de óbitos a cada ano, representando cerca de 12% de todas as causas de morte no mundo. Embora as maiores taxas de incidência de câncer sejam encontradas em países desenvolvidos, dos dez milhões de casos novos anuais, cinco milhões e meio são diagnosticados nos países em desenvolvimento (GUERRA *et al*., 2005).

O câncer do colo do útero ou câncer cervical é a segunda malignidade mais comum em mulheres e o sétimo de maior incidência no mundo com 493 mil novos casos por ano, com taxa de fatalidade de 50 a 55% e uma taxa de sobrevivência de 61% em países desenvolvidos e 41%, em média, nos países em desenvolvimento. Há uma maior incidência na África Subsaariana, Melanésia, América Latina e Caribe, Ásia Central e Sudeste da Ásia. A sobrevida mundial média estimada para cinco anos do câncer do colo do útero é de 49%. Nos países desenvolvidos esta média está entre 59 e 69%, enquanto que naqueles em desenvolvimento é menor que 49%, pois os casos são encontrados relativamente em estágio avançado (Figura 2) (CASTELLETTI, 2006).

Cerca de 80% dos casos de câncer de colo do útero ocorrem em países em desenvolvimento, sendo que em alguns destes países é o câncer mais prevalente, mesmo quando ambos os sexos são analisados conjuntamente. Contrariamente ao câncer de mama, que apresenta incidência maior em regiões brasileiras mais ricas, o de colo uterino está mais associado a regiões de baixo nível socioeconômico. No Brasil, o câncer cervical ainda continua sendo um grande problema de saúde pública, demonstrando uma variação na incidência entre várias regiões do país, de 26.72/105

no sul para 43.85/105 no norte (NAUD *et al*., 2006). Em São Paulo, por exemplo, mesmo com elevado potencial econômico, taxas de incidência elevadas (35,1% por 100.000 mulheres) são observadas (INCA, 2003).

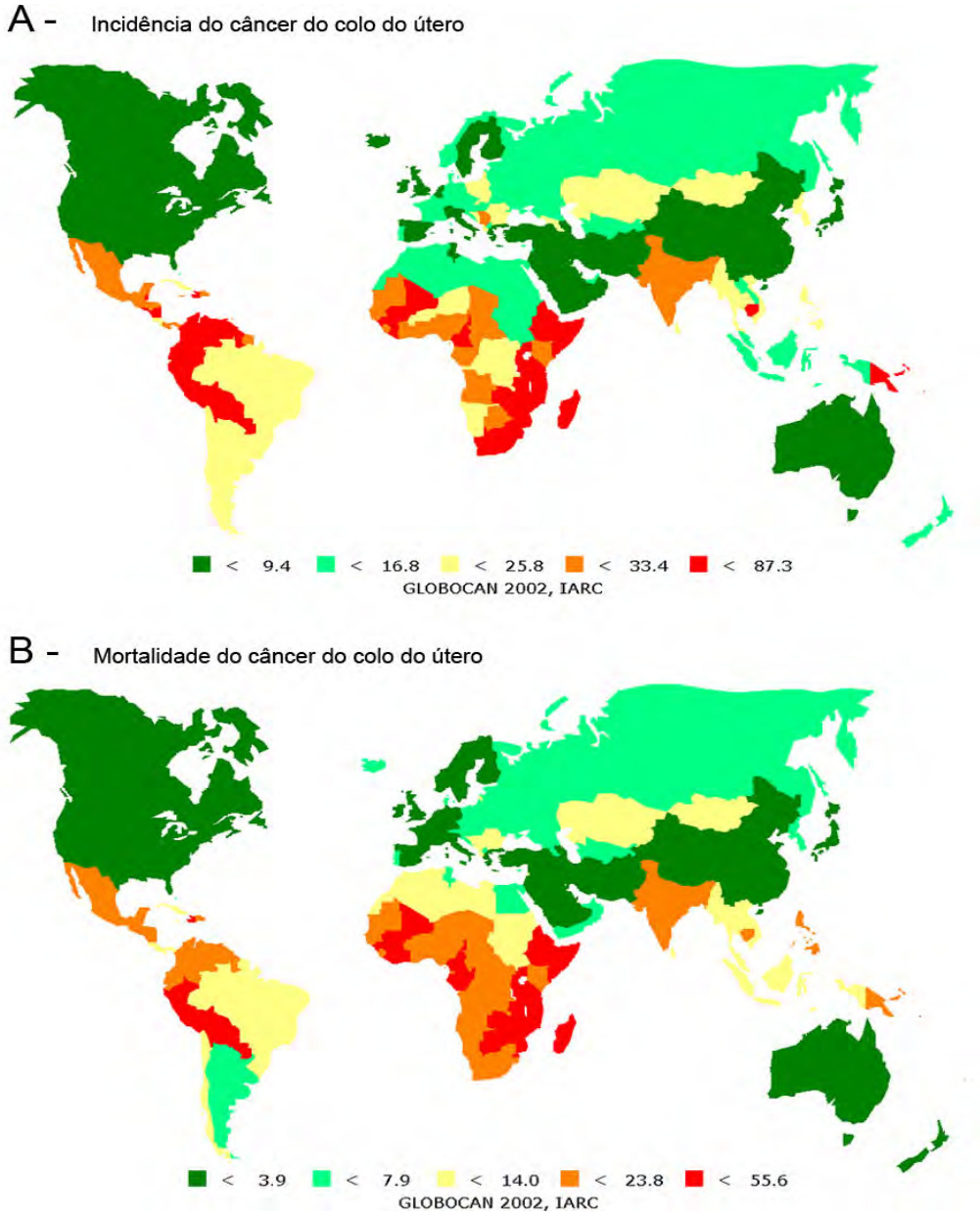

Figura 2. Incidência (A) e mortalidade (B) por câncer do colo do útero ajustado por idade para 100.000 mulheres. Dados baseados no Globocan 2002, IARC (http://www-dep.iarc.fr). Fonte: (CASTELLETTI, 2006).

O trato genital feminino consiste de um útero com cérvix, e duas tubas de Falópio, cada qual terminando próxima a um ovário. A cérvix uterina é revestida por dois tipos diferentes de epitélio, glandular no canal endocervical, e pavimentoso na ectocérvix, que se continua com o revestimento epitelial pavimentoso na vagina. A ectocérvix é composta de epitélio pavimentoso estratificado. A camada mais profunda é a basal, camada regenerativa de pequenas células que se coram intensamente e arranjadas perpendicularmenre à membrana basal. Acima desta, comparecem as camadas parabasais, de células intermediárias, incluindo as células naviculares e, na superfície, a camada de células superficiais (Figura 3) (MCKEE, 2001).

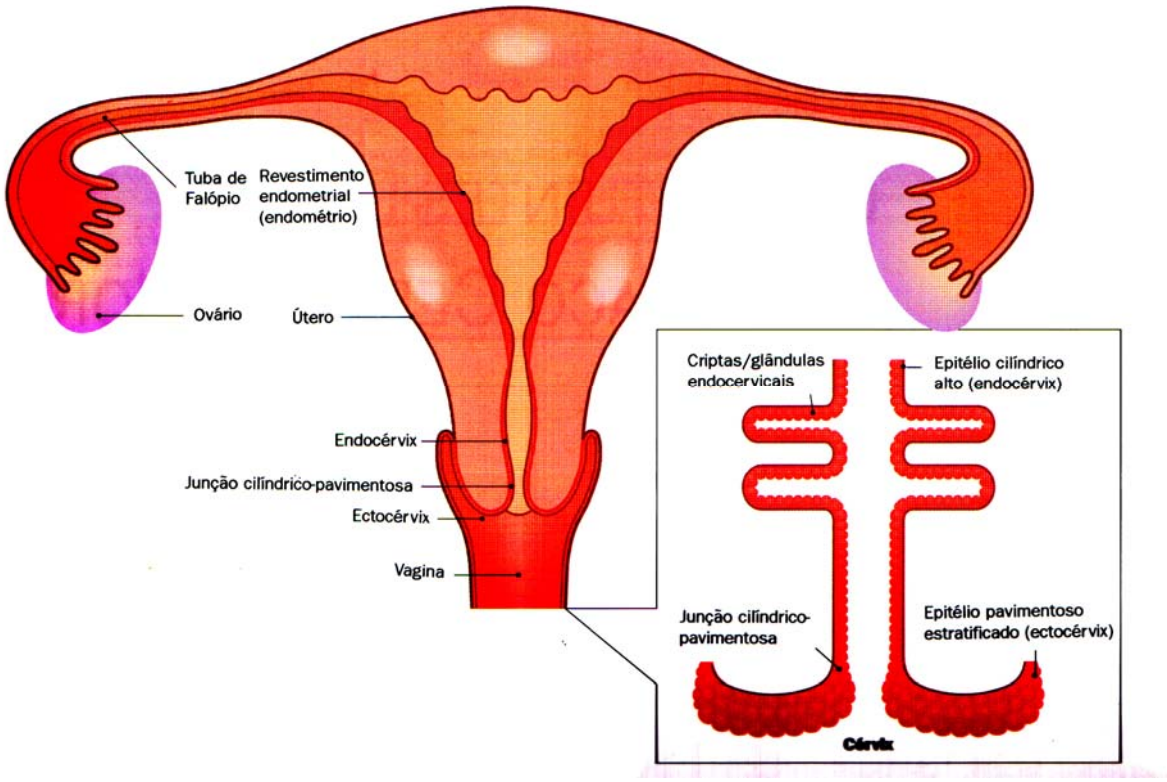

Figura 3. Diagrama do trato genital feminino. Fonte: (MCKEE, 2001).

Há considerável evidência de que o HPV é um fator de risco primário no desenvolvimento do câncer cervical, assim desempenhando uma função central na carcinogênese. Entretanto, a presença isolada do HPV não é suficiente para o desenvolvimento do carcinoma cervical, pois a maioria das infecções causadas pelo HPV regride espontaneamente e com nenhuma manifestação clínica. (ZEFERINO & DERCHAIN, 2006). Além disso, Muñoz *et al*. (2006) observaram que algumas mulheres portadoras da infecção, pelo HPV, não desenvolveram câncer cervical. Portanto, há três grupos de cofatores relevantes que podem estar relacionados à progressão do câncer cervical, tais como: (1) cofatores ambientais e exógenos, incluindo contraceptivos hormonais, tabagismo, atividade sexual precoce, consumo excessivo de álcool, dieta deficiente em vitamina A, multiparidade e exposição prévia às ISTs, como exemplo, infecção bacteriana por *C. trachomatis*; (2) cofatores virais, como vírus herpes simples tipo 2 (HSV-2), vírus da imunodeficiência humana (HIV), assim como outros tipos de HPV e (3) cofatores do hospedeiro, como aspectos imunogenéticos (SANTOS *et al*., 2002).

Desde 1960, uma redução significativa da incidência do câncer cervical foi observada em países com programas de triagem organizados, Papanicolau. Entretanto, em países em que inexistem programas de triagem coordenados, a incidência de câncer cervical permanece elevada e/ou estável (VIZCAINO *et al*., 2000; NYGARD *et al*., 2002). Enquanto que, nas décadas de 70 e 80 surgiram as primeiras evidências da relação entre infecção viral e desenvolvimento da neoplasia invasora do colo uterino e, em meados dos anos 90, a relação direta entre a associação desse câncer com o HPV foi evidenciada (NICOLAU, 2003).

No ano de 1998, motivado pelos dados estatísticos, retrospectivos e prospectivos, o Ministério da Saúde instituiu o Programa Nacional de Combate ao Câncer do Colo Uterino - PNCC. Dentro dos critérios para diagnósticos estabelecidos, o PNCC definiu como lesões intraepiteliais de baixo grau (LBG) as alterações citológicas induzidas pelo HPV, atipias de células escamosas de caráter desconhecido (ASCUS), atipias de células glandulares de significado indeterminado (AGUS) e neoplasia intra-epitelial grau I (NIC I); as alterações NIC II e III (neoplasias intraepiteliais de graus II e III) foram classificadas como lesões intraepiteliais de alto grau (LAG) (ROBERTO NETO *et al.*, 2001).

De acordo com o sistema de classificação de Bethesda, o termo lesão intraepitelial de baixo grau (LSIL, *low grade squamous intraepithelial lesion*) corresponde ao termo neoplasia/displasia intraepitelial cervical (NIC I) e a coilocitose, induzida pelo HPV. Enquanto que, a lesão intraepitelial escamosa de alto grau (HSIL, *high grade squamous intraepithelial lesion*) inclui a displasia moderada ou NIC II e a displasia severa ou NIC III (NICOL *et al.*, 2005).

Diversos estudos demonstraram que o maior risco para desenvolver câncer de colo uterino é a não realização de exames citopatológicos rotineiramente. Contudo, é importante lembrar que não há método de rastreamento, diagnóstico ou terapêutico, em medicina, que tenha 100% de certeza ou sucesso, pois algumas mulheres ainda desenvolverão câncer de colo uterino, apesar de aderirem, adequadamente, aos protocolos de investigação (RIVOIRE, 2001).

2.3 Aspectos Epidemiológicos do Câncer Cervical

Apesar do reconhecimento da existência de associação entre o HPV e o câncer ter ocorrido há 15 anos, esta doença continua sendo uma das principais preocupações das organizações mundiais da saúde, em particular nos países em desenvolvimento (MUÑOZ *et al*., 2004) e ocupa lugar de destaque na comunidade científica integrando grandes áreas como a virologia, oncologia, imunologia, biologia molecular e genética molecular (MOTOYAMA *et al*., 2004).

Com aproximadamente 500 mil casos novos por ano no mundo, o câncer do colo do útero é o segundo tipo de câncer mais comum entre as mulheres, sendo responsável pelo óbito de, aproximadamente, 230 mil mulheres por ano. Sua incidência é cerca de duas vezes maior em países menos desenvolvidos comparado com os mais desenvolvidos. A incidência por câncer do colo do útero torna-se evidente na faixa etária de 20 a 29 anos e o risco aumenta rapidamente até atingir seu pico geralmente na faixa etária de 45 a 49 anos. Quase 80% dos casos novos ocorrem em países em desenvolvimento onde, em algumas regiões, é o câncer mais comum entre as mulheres (INCA, 2008).

Em 1996, *World Health Organization* (WHO), a *European Research Organization on Genital Infection and Neoplasia e o National Institute of Health Consensus Conference on Cervical Cancer*, reconheceram que os HPV são importantes e fundamentais causas de câncer cervical, o qual, segundo Ries (2001) representa o terceiro tipo de neoplasia mais freqüente nos Estados Unidos, perdendo apenas para o câncer de pele e mama, respectivamente (HARRO *et al.,* 2001).

De acordo com a WHO, cerca de 270.000 mortes ocorrem todo ano no mundo inteiro induzidas pelas doenças clínicas de etiologia associada ao HPV. Dessas, aproximadamente 40.000 ocorrem nas regiões mais desenvolvidas do mundo, incluindo Europa, Estados Unidos, Japão, Nova Zelândia e Austrália. Mais de 230.000 mortes ocorrem em regiões menos desenvolvidas, incluindo o Brasil (ZEFERINO & DERCHAIN, 2006).

No Brasil, as estimativas para o ano de 2008 e válidas também para o ano de 2009, apontam que ocorrerão 466.730 casos novos de câncer. Os tipos mais incidentes, à exceção do câncer de pele do tipo não melanoma, serão os cânceres de próstata e de pulmão no sexo masculino e os cânceres de mama e de colo do útero no sexo feminino, acompanhando o mesmo perfil da magnitude observada no mundo. O número de casos novos de câncer do colo do útero esperados para o Brasil no ano de 2008 é de 18.680, com um risco estimado de 19 casos a cada 100 mil mulheres (Figura 4) (Tabela 1). Sem considerar os tumores de pele não melanoma, o câncer do colo do útero é o mais incidente na região Norte (22/100.000). Nas regiões Sul (24/100.000), Centro-Oeste (19/100.000) e Nordeste (18/100.000) (Tabela 2) ocupa a segunda posição mais freqüente e no Sudeste (18/100.000) a quarta posição (INCA, 2008).

Em países subdesenvolvidos, a sobrevida média é de cerca de 49% após cinco anos, porém estima-se que há uma redução de cerca de 80% da mortalidade, por este câncer, que pode ser alcançada através do rastreamento de mulheres na faixa etária de 25 a 65 anos com o teste de Papanicolau e que lesões precursoras com alto potencial de malignidade ou carcinoma "*in situ*" (INCA, 2008).

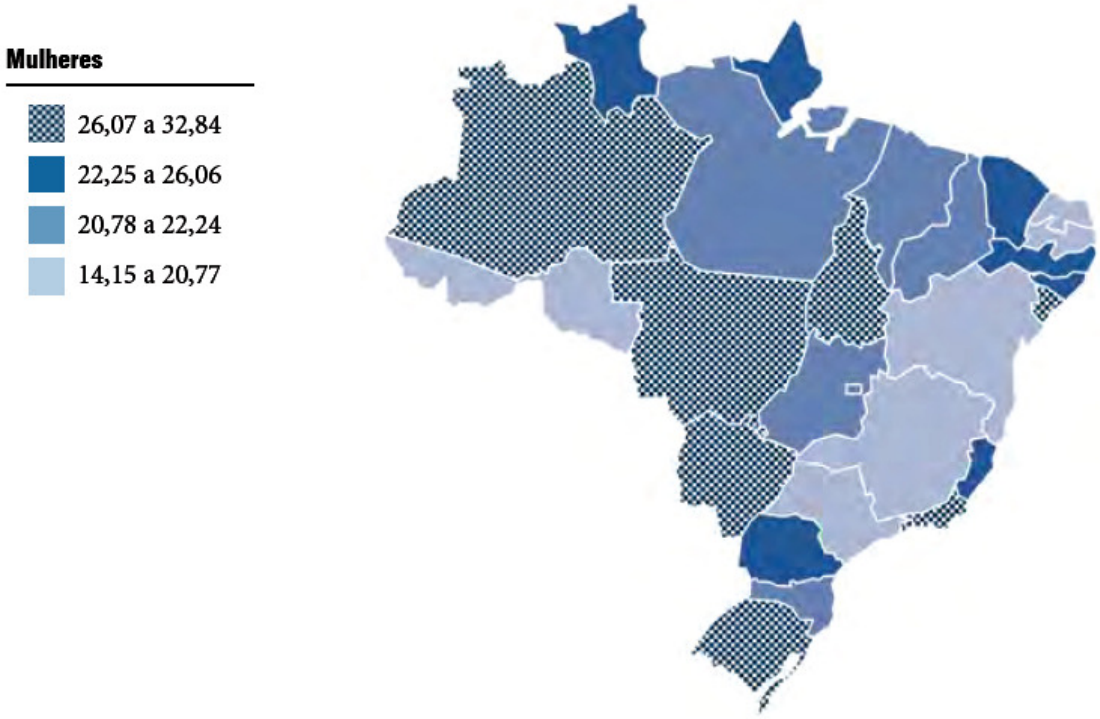

Figura 4. Representação espacial de taxas brutas de incidência por 100 mil mulheres, estimadas para o ano de 2008, segundo a Unidade da Federação (neoplasia maligna do colo do útero e do útero, porção não especificada (INCA, 2008).

Tabela 1. Estimativas para o ano 2008 das taxas brutas de incidência por 100.000 e de número de casos novos por câncer, em mulheres, segundo localização primária no Brasil consolidado (INCA, 2008).

Localização Primária Neoplasia maligna	Estimativa dos Casos Novos			
	Estado		Capital	
	Casos	Taxa Bruta	Casos	Taxa Bruta
Mama Feminina	49.400	50,71	17.400	76,04
Colo do Útero	18.680	19,18	5.620	24,49
Cólon e Reto	14.500	14,88	5.450	23,80
Traquéia, Brônquio e Pulmão	9.460	9,72	3.070	13,49
Estômago	7.720	7,93	2.380	10,30
Leucemias	4.320	4,44	1.340	5,89
Cavidade Oral	3.780	3,88	1.140	4,83
Pele Melanoma	2.970	3,03	930	3,69
Esôfago	2.650	2,72	620	2,30
Outras Localizações	62.270	63,93	22.530	98,39
Subtotal	**175.750**	**180,43**	**60.480**	**264,11**
Pele não Melanoma	59.120	60,70	14.140	61,73
Todas as Neoplasias	**234.870**	**241,09**	**74.620**	**325,77**

Tabela 2. Estimativas para o ano 2008 das taxas brutas de incidência por 100.000 e de número de casos novos por câncer, em mulheres, da região Nordeste (INCA, 2008).

Localização Primária Neoplasia maligna	Estimativa dos Casos Novos			
	Estado		Capital	
	Casos	Taxa Bruta	Casos	Taxa Bruta
Mama Feminina	7.630	28,38	3.080	51,70
Colo do Útero	4.720	17,58	1.420	23,71
Cólon e Reto	1.550	5,78	730	12,20
Traquéia, Brônquio e Pulmão	1.410	5,26	580	9,78
Estômago	1.470	5,45	450	7,52
Leucemias	830	3,15	280	5,04
Cavidade Oral	970	3,62	260	4,36
Pele Melanoma	230	0,89	120	1,48
Esôfago	420	1,62	140	1,82
Outras Localizações	8.990	33,43	5.020	83,87
Subtotal	**28.220**	**104,94**	**12.080**	**201,82**
Pele não Melanoma	14.140	52,58	3.090	51,86
Todas as Neoplasias	**42.360**	**157,48**	**15.170**	**253,47**

A mortalidade brasileira, por câncer cervical, é muito elevada ao ser comparada com a do Reino Unido, mesmo que as mulheres brasileiras com idade superior a 45 anos tenham sido submetidas a um exame de prevenção do câncer cervical. Infelizmente, os métodos não invasivos comumente utilizados em países em desenvolvimento similares àqueles empregados em países desenvolvidos, dependem do seguimento de programas para a redução de mortalidade cervical e que, na maioria das vezes, requerem de um custo elevado (NDISANG *et al.*, 2006).

A distribuição dos sorotipos do HPV mostra uma notável variação geográfica e riscos distintos para a indução dos precursores do câncer cervical para a progressão da doença (ZUR HAUSEN, 2002; MUÑOZ *et al.*, 2003). Existem evidências de que a epidemia do HPV tem aumentado progressivamente durante as últimas décadas, sendo que com as infecções relacionadas pode-se estimar que, no momento, é a doença

sexualmente mais transmitida em nível mundial. Além disso, estudos recentes têm documentado um aumento da prevalência de cânceres genitais em áreas como América Latina e Ásia. Cada câncer, especialmente o carcinoma cervical, tem sido controlado na América do Norte, com menos de 10.000 casos por ano. Não obstante, em países da América Latina como Brasil, México, Colômbia, Venezuela, dentre outros, ainda estão reportando elevadas incidências de câncer genital anualmente (FISCHER, 1994).

No Brasil, um país com proporções continentais, estudos da prevalência do HPV em câncer cervical foi observada em quatro das suas cinco regiões. Esses estudos publicados demonstram que aproximadamente 40.000 novos casos de câncer cervical têm sido diagnosticados, a cada ano, e que o HPV 16 é o tipo predominante em regiões do Norte, Nordeste, Sudeste e Sul do país, ressaltando-se que além das variações regionais outros tipos de HPV poderão existir. Este fato ainda é a principal causa de morte de câncer em mulheres brasileiras, com 8.000 casos de óbitos por ano (CAVALCANTI *et al*. 1994; ELUF-NETO *et al*. 1994; GONÇALVES *et al*. 1999; NORONHA *et al*., 1999; LORENZATO *et al*. 2000).

Essas razões factíveis têm sido significantes para explicar as variações na incidência entre as diferentes regiões brasileiras. Primeiramente, mulheres que habitam em diferentes regiões estão distintamente expostas aos fatores de risco conhecidos para a progressão da doença (HPV oncogênico). Além disso, a história natural dos precursores do câncer pode ser diferente nessas populações. A terceira explicação pode ser uma eficácia aumentada na detecção de precursores do câncer e a sua erradicação em regiões de baixa incidência (FRANCO *et al*., 1996; MILLER *et al*., 2000).

No Nordeste e Centro-Oeste brasileiros, o HPV tipo 33 e 31 são os tipos que apresentam maior incidência depois do HPV-16, enquanto que nas regiões Norte, Sudeste e Sul, o tipo 18 é o segundo mais freqüente entre os casos de câncer de colo de útero (BOSCH *et al*., 1995; RABELO-SANTOS *et al*., 2003). De acordo com uma distribuição geográfica, pelo menos 15 tipos de HPV, considerados de alto risco, têm sido associados com câncer do colo do útero, sendo os tipos 16, 18, 45, 31 e 33 os mais freqüentes (MUÑOZ *et al*., 2003).

No Estado de Pernambuco, em 2008 está estimada uma taxa de incidência de câncer de colo do útero na população feminina é de 1020 novos casos/100 000 mulheres, com taxa bruta de 22,73, enquanto que na capital é de 210 novos casos e com taxa bruta de 22,26 (Tabela 3).

Tabela 3. Estimativas de Pernambuco, para o ano 2008, das taxas brutas de incidência por 100 mil e de número de casos novos por câncer, em mulheres, segundo localização primária (INCA, 2008).

Localização Primária Neoplasia maligna	Estimativa dos Casos Novos			
	Estado		Capital	
	Casos	Taxa Bruta	Casos	Taxa Bruta
Mama Feminina	2.010	44,82	730	87,90
Colo do Útero	1.020	22,73	210	25,26
Cólon e Reto	420	9,45	180	21,82
Traquéia, Brônquio e Pulmão	310	7,01	110	12,92
Estômago	280	6,22	80	9,11
Leucemias	170	3,86	50	6,47
Cavidade Oral	180	3,94	30	3,97
Pele Melanoma	50	1,08	20	2,14
Esôfago	90	2,09	20	2,05
Outras Localizações	2.070	46,16	1.110	133,05
Subtotal	**6.600**	**147,18**	**2.540**	**304,46**
Pele não Melanoma	3.450	76,98	420	50,94
Todas as Neoplasias	**10.050**	**224,04**	**2.960**	**354,81**

*Números arredondados para 10 ou múltiplos de 10.

No Brasil, em infecções causadas por HPV do tipo 18, aproximadamente 30% das lesões cervicais são detectadas e 39% são de cânceres cervicais em pacientes residentes em Recife-PE, assim explicando o baixo prognóstico de LSIL em nosso país. Essa situação é contrária a da Europa, pois os índices não excedem 10%. Dessa forma, Recife-PE, uma cidade localizada no Nordeste brasileiro apresenta elevados índices de câncer cervical no Brasil (VILLA, 1997).

Estudos para análise epidemiológica utilizando teste de DNA para HPV em mulheres assintomáticas estimou a prevalência da infecção pelo vírus entre 2 a 44% no total das pacientes analisadas (GJOOEN *et al.,* 1996; FRANCO *et al.,* 1997; BASEMAN *et al.,* 2005; HERRERO *et al.,* 2005). Essa ampla variação na prevalência é largamente explicada pelas diferenças de idade nas populações envolvidas nesses estudos, bem como pela sensibilidade molecular dos testes empregando a tecnologia de DNA. Ademais, acrescentando, o *National Disease and Therapeutic Index* contabilizou o número inicial de consultas clínicas para diagnóstico e tratamento de lesões induzidas pelo HPV, nas instituições privadas dos Estados Unidos que cresceu de 56.000 em 1966 para mais de 350.000 no ano de 1987 (CENTERS FOR DISEASE CONTROL AND PREVENTION, 2003).

Há uma forte evidência que sugere que infecções causadas por HPV e *C. trachomatis* desempenham um papel central na etiologia da neoplasia intraepitelial cervical (NIC) (MUÑOZ *et al*., 1996, STRAND *et al*., 1998), e, subseqüentemente, o câncer cervical (WALLIN *et al*., 1999, SMITH *et al*., 2002). Observações clínicas realizadas desde 1970 indicam que a infecção genital por *C. trachomatis* é estudada como um cofator no desenvolvimento e/ou gênese de NIC e outras alterações celulares significativas em mulheres com história de infecção por HPV, resultando também em

cervicites mucopurulentas crônicas, uretrites, doença inflamatória pélvica (18 a 24%) e endometriose (6 a 21%) (TAMIM *et al.,* 2002).

A *C. trachomatis* é uma das causas de ISTs, mais freqüente nos países industrializados, e capaz de acarretar sérias complicações aos indivíduos. Em decorrência da maioria de pacientes, infectados pela bactéria, ser assintomática (50 a 70%), uma proporção significativa dos mesmos permanece sem diagnóstico, assim podendo desenvolver severidades a partir da infecção primária, contudo, estima-se que menos de 10% das infecções prevalentes são diagnosticadas (HONEY *et al.,* 2002).

A Organização Mundial de Saúde (OMS) calculou a ocorrência de cerca de 89 milhões de novos casos de infecção por este patógeno no ano de 1995 e 92 milhões em 1999 (WHO, 1995; PEELING & BRUNHAM, 1996). Nos Estados Unidos, estima-se o surgimento de cerca de quatro milhões de novos casos anuais de *C. trachomatis,* com o prejuízo adicional de 50.000 mulheres que se tornam inférteis em decorrência da infecção. A prevalência da infecção por *C. trachomatis*, em adultos entre 18 e 26 anos, é de 4,19% (WASHINGTON *et al*., 1987).

Nos Estados Unidos, desde 1990, vigora a lei de notificação compulsória de casos de infecção por *C. trachomatis*. No ano de 2002, foram reportados 834.555 casos (296,5 casos/100.000 habitantes), sendo que o aumento do número de casos reportados, ano após ano, desde 1987, refletiu por um lado, a expansão dos programas de triagem e de notificação nesse país com o desenvolvimento e uso de testes de diagnósticos mais sensíveis e, por outro lado, a verificação das taxas de incidência da infecção (CENTERS FOR DISEASE CONTROL AND PREVENTION, 2002).

Em alguns países da Europa a prevalência da infecção por *C. trachomatis*, é em torno de 4,6%, enquanto que no Brasil, as taxas recentes de infecção na população em

geral são ligeiramente superiores às citadas anteriormente, 6,1% na população em geral, e 9% em gestantes (LA MONTAGNE *et al*., 2004).

A infecção por *C. trachomatis* também é a mais comum IST de etiologia bacteriana no Reino Unido. Estima-se que aproximadamente 10,3% das mulheres e 13,3% dos homens com menos de 25 anos e residentes do Reino Unido sejam infectados pela *C. trachomatis*. Em outros países, a sua infecção genital também está presente, como na Dinamarca que, no ano de 1998, foram diagnosticados 13.000 novos casos sendo 75% em mulheres, correspondendo a uma prevalência de 4,5% (JENSEN *et al*., 2003). Na Grécia a prevalência da doença em mulheres, com idade entre 18 a 35 anos, foi estimada a cerca de 7 a 10,6% (ARKOULIS *et al*., 1989; KOSTOULA *et al*., 1994). Da mesma forma, nos anos 90 foram determinadas prevalências na Itália (2,7%), na França (4,9%), na Holanda (5,4%), na Hungria (6,2%) e na Irlanda (8,0%) (SPILIOPOULOU *et al*., 2005).

No Brasil, a infecção por *C. trachomatis* é uma patologia de notificação não compulsória. Alguns estudos de prevalência e incidência vem sendo conduzidos de forma isolada em populações e regiões distintas, utilizando-se da metodologia dos testes moleculares para a detecção da mesma, o que permite a realização de triagens populacionais. Estudo de base populacional realizado em Vitória-ES, com mulheres adolescentes, sexualmente ativas, foi constatada uma prevalência de 12,2%, enquanto que, na cidade de Manaus, a prevalência estimada em 121 amostras de pacientes femininas oriundas de um serviço de IST foi de 20,7% (SANTOS *et al*., 2003; MIRANDA *et al*., 2004).

Codes e colaboradores (2002) revelam uma prevalência da infecção por *C. trachomatis* em 11,4% das pacientes atendidas pela Clínica de Planejamento Familiar

do Estado da Bahia, das quais 60% eram assintomáticas e não usuárias de métodos anticonceptivos. Em outra análise, foi observada uma variação na prevalência de acordo com o nível de instrução e nível sócio-econômico que variou de 11,4% (geral), 17,1% (nível de instrução secundário) e de 12,9% (nas comunidades pobres) (CODES *et al.,* 2006).

Golijow e colaboradores (2005) realizaram um estudo diferente comparativo em La Plata, na Argentina para avaliar a prevalência do HPV e *C. trachomatis* em 279 mulheres, pelo método da pesquisa de DNA, destas 79 com citologia oncótica normal e 200 com anormal. Observou-se que a prevalência por *C. trachomatis* foi de 11% nas pacientes com citologias oncóticas normais, ao passo que nas pacientes com citologias oncóticas anormais, com lesão de alto grau, houve um incremento para 47% e de 20% nos casos carcinoma escamoso, enquanto que a presença do HPV variou de 30% nas pacientes com citologias oncóticas normais, para 99-100% nas pacientes com citologias oncóticas anormais, assim demonstrando que a presença de *C. trachomatis* pode estar associada com as LBG e LAG, precursoras do câncer cervical.

A incidência e mortalidade de câncer cervical têm diminuído, em parte pelo diagnóstico precoce e tratamento de lesões precursoras do câncer cervical (IVOR, 2004). Diversos estudos demonstraram que o maior risco para desenvolver câncer de colo uterino é a não realização de exames citopatológicos rotineiramente. Contudo, é importante lembrar que não há método de rastreamento, diagnóstico ou terapêutico, em medicina, que tenha 100% de certeza ou sucesso. Assim, algumas mulheres ainda desenvolverão câncer de colo uterino, apesar de aderirem adequadamente aos protocolos de investigação (RIVOIRE, 2001).

2.4 Histórico do Papilomavírus Humano (HPV)

O desenvolvimento das pesquisas focando a história natural da infecção pelo HPV teve uma contribuição maciça do virologista alemão Harald zur Hausen e outros pesquisadores, que a partir de dados experimentais, analisaram o possível papel do HPV no câncer cervical entre os anos 1974 e 1976 (FRANCO, 1995; BURD, 2003).

Durante os últimos 25 anos, a compreensão a respeito de papilomaviroses, em particular, o HPV tem crescido drasticamente (MUÑOZ *et al*., 2003). O mesmo foi o primeiro vírus tumorigênico a ser transmitido experimentalmente de um hospedeiro para outro, em 1894, quando Licht inoculou, em si próprio, material da verruga de seu irmão, verificando o aparecimento de uma lesão no local de inoculação. Entretanto, apenas em 1970, que Ciuffo verificou a etiologia viral dessas lesões cutâneas (SANTOS *et al*., 2002). Nesse mesmo ano, o primeiro (HPV)-DNA foi encontrado em câncer cervical. Subseqüentemente, o HPV foi encontrado em 90-95% das pacientes com câncer cervical, sendo os tipos mais freqüentes HPV-16 (50%), HPV-18 (12%), HPV-45 (8%) e HPV-31(5%) (BOSCH *et al*., 1995). De fato, os primeiros tipos virais foram apenas isolados e, posteriormente, clonados diretamente de biópsias da cérvix no início da década de 80, particularmente em 1983 e 1984, para o HPV-16 e HPV-18, respectivamente (Tabela 4) (ZUR HAUSEN, 2002).

Tabela 4. Cronologia dos principais eventos/fatos envolvidos no estudo do papilomavírus humano e o câncer cervical. Fonte: (BRANDÃO, 2007).

Ano	Descobertas, eventos ou fatos principais
1974-76	Início do postulado entre a infecção do HPV e o câncer cervical.
1977	Isolamento do HPV em verrugas genitais e papiloma laringeal
1981	zur Hausen detecta DNA de HPV em neoplasias cervicais
1983	Isolamento e clonagem de HPV-16 de biópsia da cérvix
1984	Isolamento e clonagem de HPV-18 de biópsia da cérvix
1985	Estrutura e transcrição do HPV em células com carcinomas
1987	Primeiros grandes estudos epidemiológicos → Relação entre a infecção e a idade
1989	Proposição do modelo das oncoproteínas E6/E7 na transformação celular
1991	IARC† e WHO‡ declaram oficialmente a associação entre o HPV e câncer cervical

† Agência Internacional para Pesquisa em Câncer (IARC, "International Agency for Research on Cancer");

‡ Organização Mundial da Saúde (WHO, "World Health Organization").

Em 1996, foi oficialmente declarado e reconhecido no segundo "Workshop Internacional de Epidemiologia" do câncer cervical e HPV, realizado pela Agência Internacional para Pesquisa em Câncer e pela Organização Mundial da Saúde, a associação entre a infecção pelo HPV e o câncer cervical. Tal afirmação foi baseada em dados epidemiológicos e evidências laboratoriais alcançadas na época (MOTOYAMA *et al.*, 2004).

Desde a caracterização dos primeiros tipos de HPV cutâneos, ao longo da década de 1970, que mais de 200 tipos de papilomaviroses (PV) têm sido isolados e caracterizados, em base do seqüenciamento de DNA, assim demonstrando diferenças genômicas entre eles. Desses, oitenta e cinco (85) são genótipos de HPV bem caracterizados, enquanto que cento e vinte (120) são parcialmente caracterizados como

potenciais novos genótipos (ZUR HAUSEN, 1999). Após mais de 20 anos de investigações sobre a associação existente, entre o HPV e o carcinoma escamoso cervical, poucas dúvidas restam a respeito do papel central desse vírus na etiopatogênese do câncer cervical (VILLA *et al*., 1997).

2.4.1 Organização Estrutural do HPV

A família dos papovavírus (*Papovaviridae*) inclui os poliomavírus e os papilomavírus (SPENCE & JOHNSTON, 2003). Esses pequenos vírus, com 50 a 55nm de diâmetro, não-envelopado e constituído de capsídeos icosaédricos com 72 capsômeros (Figura 5), infectam e se proliferam em células basais, sendo a sua produção viral associada às camadas de diferenciação do epitélio, o que indica que a diferenciação celular e proliferação do vírus estão relacionadas (DUFFY *et al*., 2003).

Figura 5. Desenho esquemático da estrutura icosaédrica do HPV.
(uk.news.yahoo.com/325/photo/hpv-virus.html)

O genoma do HPV, consiste de uma fita dupla de DNA circular, cerca de 8000 pb associadas com histonas (Figura 6). Normalmente esse genoma é mantido em sua forma super-enovelada e apresenta oito códigos abertos de leitura (*open reading frames* (*ORFs*)), que se dividem em três regiões: (i) região constituída de 400 a 1000 pb, a qual tem sido referida como região não-codificante ou região longa de controle (*long control region*-LCR). Esta região contém seqüências silenciadoras que regulam a replicação do DNA pelo controle da transcrição dos ORFs e também permite um elevado grau de variação no genoma viral. (ii) a segunda é uma região precoce (*early region*), constituída de ORFs E1, E2, E4, E5, E6 e E7, as quais são responsáveis por diversas funções, como replicação e transcrição viral e, a subseqüente oncogênese. (iii) a terceira região é denominada de tardia (*late region*), constituída de proteínas estruturais do capsídeo viral L1 e L2 (DELL & GASTON, 2001). Os genes L1 e L2 codificam proteínas, maior e menor, do capsídeo viral em uma proporção de 30:1 (SCHWARTZ, 2000). Além destas, existe ainda a região reguladora "*upper*" (URR), que possui vários sítios de ligação para diversos repressores e ativadores da transcrição (TUREK, 1994; PARK *et al*., 1995; BURD, 2003).

As etapas de penetração, transporte para o núcleo, desnudamento e transcrição viral não são conhecidas, mas a replicação tem início com a remoção das histonas associadas ao DNA viral e seu desenrolamento mediado pelas proteínas virais E1 e E2. Em seguida, a proteína E1 forma um complexo de replicação com proteínas celulares e a replicação do DNA progride bidirecionalmente da origem de replicação na região LCR do genoma. O DNA é então encapsidado por um processo que envolve sua associação com as proteínas histonas celulares. Uma ligação transitória com a proteína E2 guia o DNA para dentro de um agregado de proteínas L1 e L2 virais que, eventualmente,

forma o capsídeo. Pouco é conhecido sobre a montagem e liberação dos papilomavírus. A liberação das partículas parece ser passiva, não-citolítica, ocorrendo na camada cornificada do epitélio queratinizado (MURRAY *et al*., 2004).

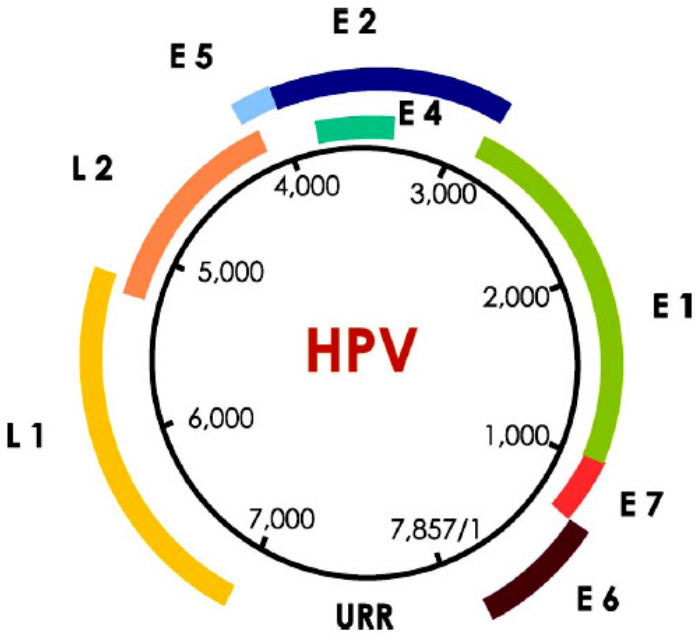

Figura 6. Representação esquemática do genoma do HPV, através da demonstração da organização de genes não-estruturais ou precoces, de genes do capsídeo (L1 e L2) e a região super-regulada (URR). Fonte: (MUÑOZ, 2006).

O DNA do HPV encontra-se normalmente na forma extra-cromossomal ou episomal nas lesões cervicais benignas. A replicação do DNA viral ocorre nas células basais da epiderme, onde permanece em múltiplas cópias de maneira estável, garantindo persistência nas células proliferativas da epiderme (GONÇALVES & DONATI, 2004). Entretanto, em várias células de câncer cervical, bem como em queratinócitos humanos transformados com HPV em testes *in vitro*, foi observado o genoma do HPV integrado ao genoma celular (PARK *et al*., 1995).

A integração do HPV de alto risco é considerada o evento mais crítico na carcinogênese cervical, e precede o desenvolvimento de anomalias cromossômicas (PETT *et al*., 2004). Este processo de integração do genoma viral parece ocorrer com maior freqüência em processos cancerosos associados ao HPV-18, do que em casos de câncer cervical relacionados ao HPV-16 (CRUSIUS *et al*., 1997). Durante esta etapa de integração do DNA viral, ocorre uma quebra da região precoce (*early region*), e conseqüentemente a perda dos genes E1, E2 e E4, responsáveis pela replicação, transcrição e maturação viral, resulta no aumento desregulado da expressão das oncoproteínas E5, E6 e E7, fator primordial para malignidade celular e implantação do tumor (Tabela 5) (PHELPS *et al*., 1988; DOORBAR *et al*., 1991).

Tabela 5. Funções dos genes existentes no Papilomavírus Humano (HPV). Modificado (HILLEMAN *et al*., 2000; SANTOS *et al*., 2002).

Gene (*ORFs*)	Função
E1	Replicação do DNA extra-cromossomal viral e regulação da transcrição viral
E2	Replicação e regulação da transcrição viral
E4	Proteína citoplasmática, maturação e liberação viral. Proteína sintetizada tardiamente e expressa nos queratinócitos diferenciados; complexa-se à citoqueratina, desencadeando seu colapso.
E3	Função desconhecida
E5	Oncoproteína de alteração do ciclo celular ciclina-quinase dependente. Capacidade de induzir transformação celular; aumenta a transdução de sinal para o receptor do fator de crescimento epidérmico (EGFR, *epidermal growth factor receptor*)
E6	Oncoproteína, supressão da p53 e imortalização.
E7	Oncoproteína, supressão da pRb e imortalização
E8	Repressão da replicação e transcrição viral, presente apenas em alguns HPVs
L1	Proteína estrutural do capsídeo viral “majoritária”
L2	Proteína estrutural do capsídeo viral “minoritária”
LCR	Região longa promotora

A E5 é uma oncoproteína de membrana, altamente hidrofóbica, com 83 resíduos de aminoácidos, vinculada ao complexo de Golgi, retículo endoplasmático e às membranas nucleares das células infectadas. A mesma pode ativar o receptor do fator de crescimento epidérmico (EGFR, *epidermal growth factor receptor*), através da subunidade pump ATPase induzindo uma diminuição da regulação do EGFR. A ativação do EGFR pode iniciar cascatas bioquímicas que conduzem a maior expressão de uma variedade de protooncogenes, capazes de estimular o rápido crescimento celular, pela inibição da expressão de genes supressores de tumor p21 (WafI/SdiI/CIPI) e p53, prejudicando o controle do ciclo celular. Pelo fato de que o gene E5 do HPV 16 pode induzir a transformação em células epiteliais, possivelmente aumentando a transdução de sinal intracelular, mediada por fatores de crescimento, recentes evidências têm identificado o alvo potencial da E5 como molécula antigênica para a produção de vacinas (SOARES & COSTA, 2003; TSAI *et al*., 2003).

Tipos específicos de HPV, principalmente os tipos 16 e 18 têm sido identificados como agentes causadores de, no mínimo, 90% de câncer na cérvix uterina e estão etiologicamente relacionados com mais de 50% de cânceres anogenitais. A integração viral promove a desregulação da expressão do gene E2, acarretando aumento da expressão das oncoproteínas virais E6 e E7 (PARK *et al.,* 1995). Essas proteínas oncogênicas dos HPV de alto risco inativam os produtos dos genes p53 e Rb, os quais representam genes supressores de tumor. A função dos genes supressores de tumor inclui a regulação do ciclo celular, bem como a regulação da resposta celular contra danos ao material genético, iniciando os reparos ao DNA, replicação, indução da apoptose e promoção da diferenciação celular (FINZER *et al*., 2002).

A oncoproteína E7 interage com pRB, uma proteína celular, produto do gene do retinoblastoma, que realiza uma importante função na regulação negativa do ciclo celular. Em conseqüência, a célula progride para a próxima etapa do clico celular, pois ocorre a liberação do fator de transcrição E2F que está acoplado a pRB, passando a estimular a expressão de uma série de genes envolvidos na proliferação celular (ZÜR HAUSEN, 1991). Este processo é intensificado pela ação da proteína E6 dos HPVs de alto risco, por ligação à p53, que culmina com a sua degradação (Figura 7) (RAPP & CHEN, 1998).

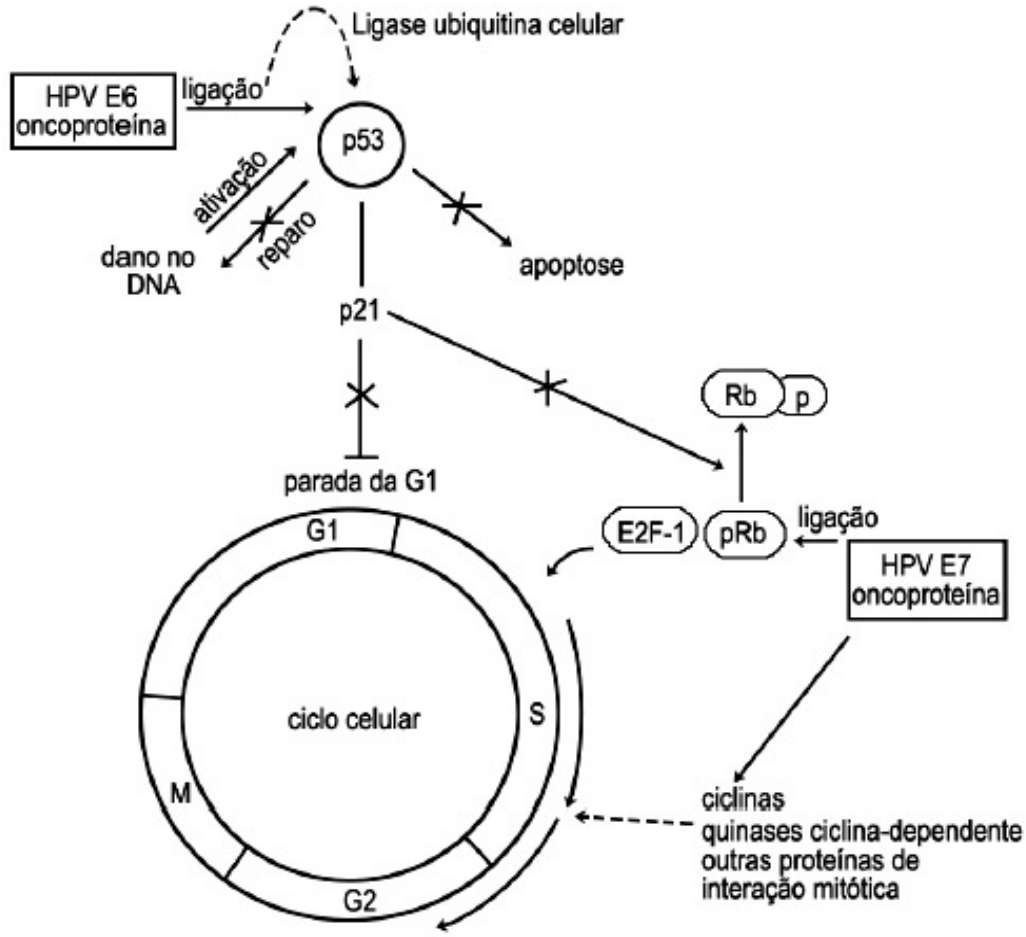

Figura 7. Esquema explicativo da patogenicidade do HPV. Os genes E6 e E7 codificam proteínas multifuncionais que se ligam primariamente às proteínas p53 e pRb, alterando o ciclo celular. Modificado (BURD, 2003).

A função da pRB é regulada durante o ciclo celular pela fosforilação. A proteína é hipofosforilada na fase G1 do ciclo celular e a fosforilação de sítios específicos aumenta aparantemente no decorrer do ciclo celular. O complexo protéico que aparece com a pRB fosforilada, na fase de síntese de DNA (fase S) incluem a ciclina (CYC) e a quinase ciclina-dependente (CDK) (provavelmente, ciclina D1 e CDK4). O complexo CDYD1/CD4 é regulado pelo inibidor de proteína p16, o qual é o produto do gene supressor de tumor no braço curto do cromossomo 9, conhecido como INK4a. No estado de hipofosforilação, a pRB se liga ao E2F, fator de transcrição regulador de proteínas. As proteínas de E2F dimerizam as proteínas DP e ativam a transcrição dos genes, incluindo àqueles envolvidos na síntese de DNA (RAPP & CORY, 1988; SCHEFFNER *et al*., 1990; HOLLAND *et al*., 1997; VENTURINI *et al*., 1999; SENIOR, 2002; SPENCE & JOHNSTON, 2003)

A E6 liga-se, seqüestra e degrada a p53, importante proteína supressora de tumor, e facilita a liberação de E2F. Acredita-se que a degradação da proteína p53 resulte no bloqueio das respostas celulares aos danos sofridos pelo DNA, permitindo assim ao acúmulo de alterações genéticas e a criação de um genótipo maligno. A expressão exagerada e a mutação de p53 são freqüentemente encontradas no carcinoma endometrial (SOARES & COSTA, 2003). A suscetibilidade da p53 ao HPV está relacionada com seu genótipo, pois o polimorfismo no códon 72 do gene codificante pode resultar na produção de uma proteína com unidades dos aminoácidos Prolina (Pro) ou Arginina (Arg). Segundo Agorastos (2000), mulheres homozigotas para Arg eram sete vezes mais suscetíveis ao carcinoma cervical do que as heterozigotas, Arg-Pro. Evidências levam a crer que a homozigose para Arg pode ser uma característica genética de alguma importância para o desenvolvimento da NIC.

2.5 Histórico da *C. trachomatis*

A *Chlamidiacea* compreende uma família de bactérias de grande importância médica, uma vez que possui como membros a *C. trachomatis*, reconhecida como agente etiológico do tracoma e de infecções do trato geniturinário, com repercussões clínicas e epidemiológicas como infertilidade, gravidez ectópica e transmissão via canal do parto para neonatos (COURA, 2005).

A palavra tracoma (do Grego *Tráchomas*) significa rugoso, áspero ou edemaciado, descrevendo a aparência da conjuntiva tarsal acometida. O tracoma é uma ceratoconjuntivite bacteriana crônica e recidivante causada pela *C. trachomatis* (sorotipos A, B, Ba e C) que costuma afetar crianças desde os primeiros meses de vida; acarreta de forma lenta: cicatrização conjuntival, entrópio, triquíase, opacidade corneana, olho seco e cegueira no adulto. Geralmente sua transmissão ocorre dentro do ambiente doméstico, de forma direta (olho para olho ou mãos contaminadas) ou indireta (vestuários e proliferação de moscas) (GARRIDO *et al*., 1999).

No Brasil, o tracoma teria chegado no século XVIII entre 1718 e 1750, com os ciganos expulsos de Portugal, desenvolvendo-se na região do Cariri no interior do Ceará, o mais antigo foco do país (LUNA *et al*., 1987; COUTO *et al*., 1997). No final do século XIX, com a chegada dos Europeus procedendo de países endêmicos do mediterrâneo (Itália e Espanha), outros focos surgiram nos Estados de São Paulo e Rio Grande do Sul, expandindo-se para outras regiões (LUCENA *et al*., 2004).

Halberstaedter e Provazek em 1907 identificaram, pela primeira vez, a bactéria *C. trachomatis*. A inclusão intracitoplasmática observada foi fortemente diagnosticada como protozoário, através do material obtido de secreções da conjuntiva, tanto em

crianças quanto em adultos. As mesmas inclusões foram observadas em material colhido do trato genital feminino. Entre os anos, 1909 e 1911, Linder demonstrou que o agente da blenorragia de inclusão foi transmissível, em primatas, assim comprovando a presença da bactéria em mães de recém-nascidos, com conjuntivite, e em parceiros sexuais dessas mulheres (SCHACHTER, 1997).

Seguidamente, durante 30 anos, houve ênfase na importância da transmissão sexual do agente como uma fonte de infecção para os recém–nascidos (RNs), de mulheres assintomáticas, entretanto portadoras da bactéria. Apenas em 1930 que o isolamento da *C. trachomatis*, causadora do linfogranuloma venéreo, foi obtido, em ratos e óvulos, sendo as demais 50 variedades de clamídia foram isoladas por cultura de células (WILFERT *et al*., 1986; SCHACHTER *et al*., 1997).

Os sorotipos da *C. trachomatis* foram classificados, em 1960, através de "teste de prevenção de toxicidade em camundongos". Neste ensaio, os animais foram submetidos à inoculação intravenosa de suspensões de *C. trachomatis*, em concentrações elevadas. Enquanto que, em 1970, Wang e colaboradores propuseram o teste de microimunofluorescência para classificar as linhagens de *C. trachomatis*, sorologicamente. Em seguida, anticorpos monoclonais têm aumentado o número de sorotipos distinguíveis e permitido um maior detalhamento dos antígenos relacionados entre as linhagens dessa bactéria (BARNES, 1989).

2.5.1 Organização Estrutural da *C. trachomatis*

A família *Chlamydiacea,* com um gênero *Chlamydia* e quatro espécies reconhecidas, sendo a *C. trachomatis* e a *C. pneumoniae* de maior importância clínica,

foi reclassificada com base na análise da seqüência de genes do RNA ribossômico 16S e 23S em dois gêneros: *Chlamydia,* que inclui a *C. trachomatis,* a *Chlamydophila*, que inclui a *C. pneumoniae* e a *C. psittaci*, dentre outras espécies de menor relevância (MAHONY *et al*., 2003).

Das três espécies de clamídia, *C. trachomatis* e *C. pneumoniae* são os patógenos humanos conhecidos. O mapeamento genético dessas duas classes tem, entretanto, confirmado a estreita similaridade entre ambas, através da presença de 70 genes em *C. trachomatis* que não existe em *C. pneumoniae*. Em decorrência de sua incapacidade de sintetizar ATP, essas espécies utilizam as fontes de energia das células hospedeiras, e, no passado, foram consideradas viroses (MANAVI, 2006).

No início, as clamídias foram consideradas como grandes vírus ou *Rickettsia,* em razão de seu pequeno tamanho e ao parasitismo intracelular obrigatório. No entanto, é possível identificá-las por meio de microscopia óptica comum, ao serem encontrados corpúsculos de Gamma-Miyagawa. A natureza bacteriana desses microrganismos é confirmada pela presença de membrana celular externa semelhante à de outras bactérias Gram-negativas, além do fato de apresentar DNA, RNA e ribossomos procarióticos típicos em seu ciclo de desenvolvimento dimórfico que ocorre na inclusão intracelular, sintetizam suas próprias proteínas, ácidos nucléicos e lipídios (Figura 8) (GUASCHINO & DE SETA, 2000).

C. trachomatis, desde 1964, é considerada o agente sexualmente transmissível mais comumente responsável pelo desencadeamento de um amplo espectro de doenças incluindo, lesões cervicais uterinas, linfogranuloma venéreo, tracoma, conjuntivite e pneumonia, em humanos (WILFERT *et al*., 1986; CAMPBELL *et al*., 1987).

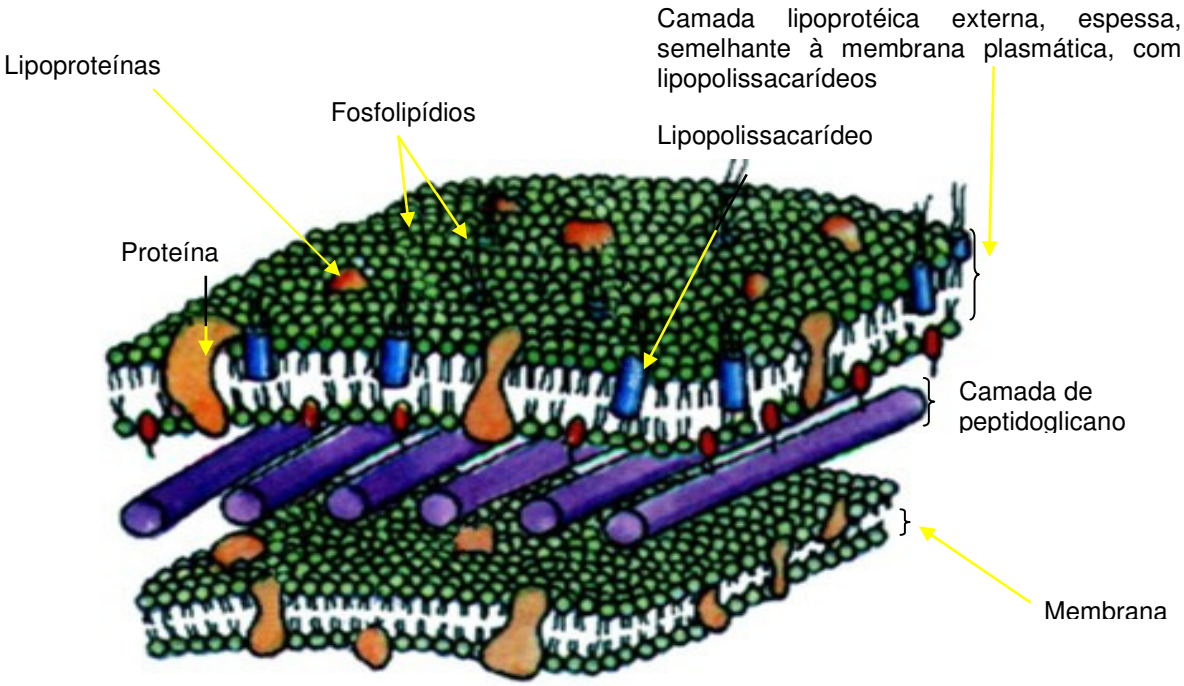

Figura 8. Esquema da parede celular da bactéria Gram-negativa. http://www.editorasaraiva.com.br/biosonialopes.

O ciclo de desenvolvimento da *C. trachomatis*, bactéria imóvel, é bifásico e sua replicação ocorre dentro de vacúolos na célula hospedeira, formando inclusões citoplasmáticas características (BARNES, 1989). A replicação apresenta um ciclo multimórfico e sem sincronismo de desenvolvimento. Dentro deste ciclo multimórfico ocorrem duas formas bem distintas: o corpo elementar (EB), 300nm de diâmetro, e o corpo reticular (RB) (HALL, 1997; SCHACHTER, 1999).

Os EBs são formas infecciosas que entram no endossoma da célula hospedeira, depois de penetrar através de receptores na superfície da célula epitelial suscetível à clamídia (Figura 9) (SEADI *et al.*, 2002). Aproximadamente oito horas após a entrada na célula, começa a replicação por divisão binária, completando-se o ciclo dentro do endossoma. No interior da célula, os EB's germinam e formam os RBs que, após uma

incubação de 7 a 21 dias, inicia a replicação a cada 3 horas. O RB (corpo reticular_forma intracelular) é maior em tamanho e mais rico em RNA, sendo a forma metabólica e não-infecciosa da clamídia. De 24 a 72 horas, o RB retorna à forma EB (corpo elementar_forma infecciosa), formando vacúolos contendo de 100 a 1.000 EB. Quando estes vacúolos substituem quase todo o citoplasma da célula hospedeira, ocorre lise e lançamento de EB para o meio extracelular, podendo dar início a um novo ciclo de infecção. Os EBs possuem a capacidade de infectar células colunares não-ciliadas e macrófagos (WARFORD, 1999).

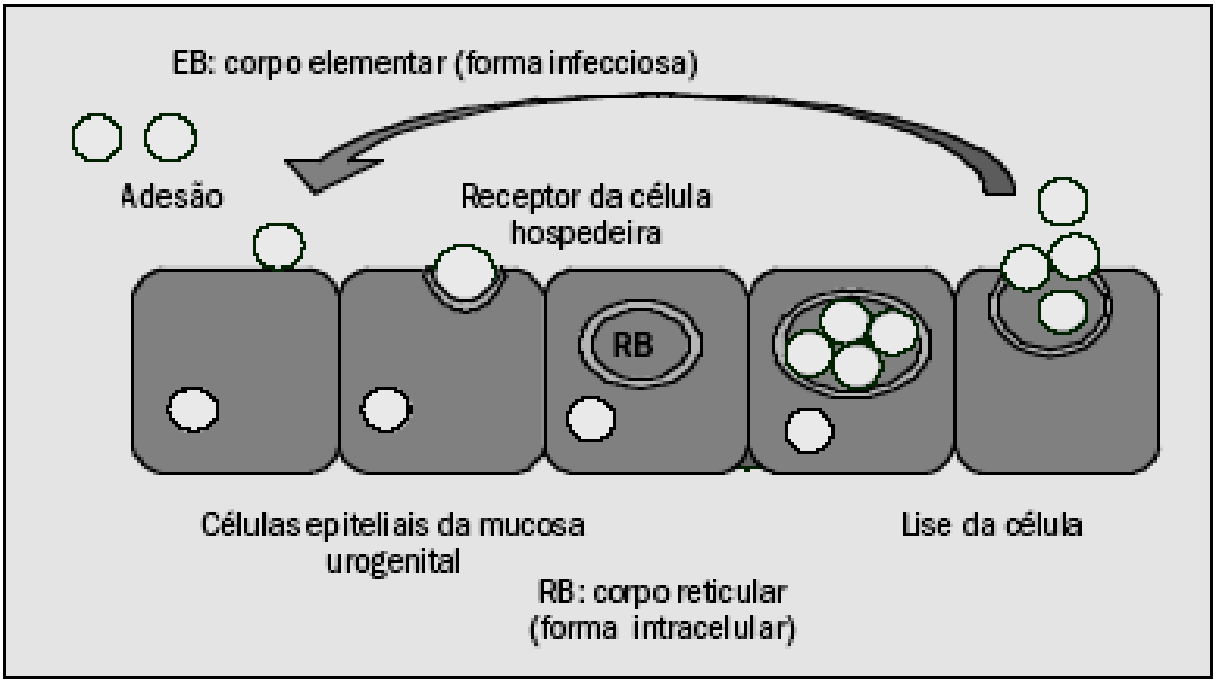

Figura 9. Esquema do ciclo de desenvolvimento da *C. trachomatis* (SEADI *et al*., 2002).

A espécie *C. trachomatis* pertence à família *Chlamydiaceae* sendo responsável pela etiogenia de patologias diferentes, associadas às biovariedades, tais como: tracoma, linfogranuloma venéreo e infecções genitais. Esse patógeno intracelular, com

15 sorotipos, pode ser classificado de acordo com as diferentes apresentações clínicas: os sorotipos A, B, Ba, C estão associados ao tracoma endêmico; L1, L2, L3 ao linfogranuloma venéreo; D, E, F, G, H, I, J, K às infecções genitais e em neonatos e do lactente, da uretrite, da cervicite, da salpingite e da epididimite (Tabela 6) (VAZ *et al*., 1999; FERREIRA & ÁVILA, 2001).

Tabela 6. Relação entre os sorotipos, o sexo acometido e as doenças causadas por *C. trachomatis* (FERREIRA & ÁVILA, 2001).

Sorotipos	Sexo Acometido	Síndrome/Doença
A, B, Ba, C	Mulheres e Homens	Tracoma e Conjuntivite
D, E, F, G,	Mulheres	Uretrite, Cervicite, Endometriose, Doença Inflamatória Pélvica (DIP), Salpingite
H, I, J, K	Homens	Uretrire, Prostatite, Epididimite
	Ambos e RNs	Conjuntivite, Pneumonia
L1, L2, L3	Mulheres e Homens	Linfogranuloma Venéreo

As infecções e doenças humanas associadas às bactérias do gênero *Chlamydia* (*C. trachomatis, C. pneumoniae* e *C. psittaci*) têm sido reconhecidas como problemas graves de saúde coletiva, e estão relacionadas às infecções oculares, como o tracoma; a infecções genitais, como as uretrites não-gonocócicas e cervicites mucopurulentas, que podem evoluir para doença inflamatória pélvica, gravidez ectópica e infertilidade e a infecções cardiorrespiratórias, como as pneumonias adquiridas em comunidade (ISHAK *et al*., 2001).

Na grande maioria dos casos, a transmissão se dá de forma direta, através do contato sexual de uma pessoa infectada para um parceiro sexual, via oral, vaginal, cervical, uretral ou retal. As bactérias, inicialmente, podem se propagar desde o sítio da infecção primária causando infecção do útero, tubas uterinas, ovário, cavidade

abdominal e nas glândulas anexas, e, no caso dos homens, pode se propagar nos testículos e próstata. Os RNs de mães infectadas e que nasceram por parto normal podem tornar-se infectados no momento que passam pelo canal vaginal e estão em risco de desenvolver conjuntivite ou pneumonia. O período de incubação para essas entidades clínicas geralmente varia de 7 a 14 dias, ou talvez mais tempo (CANADIAN SEXUAL TRANSMITED DISEASE GUIDELINES, 1998).

A maioria das infecções causadas por *C. trachomatis* é assintomática, variando de 25% a 50% em homens e de 65% até 90% em mulheres, assim dificultando o diagnóstico (*Centers for Disease Control and Prevention*, 2000; NELSON & HELFAND, 2001). Dessa forma, a infecção pode induzir complicações onde uma forma persistente deste microrganismo pode estar presente em localizações anatômicas distantes do sítio da infecção primária. Em algumas circunstâncias, a *C. trachomatis* pode se disseminar do trato urogenital para o interior da articulação ou outros locais, como a tuba uterina, causando seqüelas como artrite e infertilidade, respectivamente (BEATTY *et al*., 1994; NANAGARA *et al*., 1995; PAVONEEN & EGGERT-KRUSE, 1999; INMAN *et al*., 2000).

Nas mulheres, a infecção pode causar salpingite genital, cervicite, uretrite, endometrite, doença inflamatória pélvica, infertilidade e gravidez ectópica (HILLIS *et al.,* 1996; GAYDOS *et al.,* 1998). Quando o quadro é sintomático, não há corrimento vaginal, disúria e sangramento após a relação sexual. O foco inicial da infecção geralmente é a endocérvice (50 a 60% dos casos), e pode ocorrer em menor freqüência na uretra e reto. A ascensão do microrganismo do trato geniturinário (região endometrial) às tubas uterinas pode ocasionar dores abdominais e eventuais anormalidades menstruais (WEINSTOCK *et al.,* 1994). O paradoxo é que mesmo nas

infecções assintomáticas clinicamente há elevado risco para o desenvolvimento de formas graves de doença tubária (SCHOLES *et al.,* 1996; GEISLER *et al.,* 2001).

Os principais fatores de risco, além da própria gestação, são vida sexual ativa, baixas condições sócio-econômicas, vários parceiros e antecedentes de outras ISTs, além de que o próprio período gestacional, isoladamente, parece aumentar o risco de colonização por *C. trachomatis* (VAZ *et al*., 1999). Em relação aos RNs, a infecção pela bactéria é adquirida durante a passagem pelo canal do parto, com antecedentes maternos de ruptura prematura de membranas amnióticas, e, mesmo no pós-natal, sendo possível através do contato com a mãe (GOLDENBERG *et al*., 1997).

Uma importante estratégia de sobrevivência da *C. trachomatis* baseia-se na modulação da morte celular programada, apoptose, por um lado, ou na inibição da mesma, por outro lado. Esse mecanismo destaca o controle efetivo desses patógenos, em células infectadas, na promoção de sua própria sobrevivência, mesmo sob as mais adversas circunstâncias (BYRNE & OJCIUS, 2004).

2.6 Mecanismo de Ação do Sistema Imunológico (SI)

O SI é responsável pelo reconhecimento de substâncias estranhas, sua eliminação, remoção de células mortas e/ou danificadas, e destruição de células mutantes e cancerosas. Este está apto a gerar uma variedade enorme de células e moléculas capazes de reconhecer e eliminar especificamente substâncias "não-próprias", sem limites. As células e moléculas agem conjuntamente em uma rede dinâmica extraordinariamente adaptável semelhante aos rivais de estruturas complexas, tais como o sistema nervoso (GOLDSBY *et al*., 2002).

Funcionalmente, a imunidade possui dois caminhos principais envolvidos na eliminação de partículas estranhas: a *imunidade inata* e a *imunidade adquirida* que apresentam aspectos diferentes, tais como, sistema de reconhecimento, células envolvidas e mecanismos de ação. A imunidade inata é a primeira linha de defesa e está presente na maioria dos organismos. Os receptores das células do sistema imune inato foram desenvolvidos durante o processo evolutivo. Por outro lado, os receptores das células do sistema imune adquirido são desenvolvidos durante os rearranjos gênicos (LEHNER, 2003; SIERRA *et al.,* 2005; MGNADÓTTIR, 2006).

O ambiente é um lugar de constante agressão no qual o *Homo sapiens* deve continuamente utilizar mecanismos de defesa sofisticados, flexíveis e letais, a fim de que a ação de bactérias, vírus e parasitas eucariotos, seja minimizada. O padrão de defesa do hospedeiro está baseado em ações compreendidas entre as respostas imunes inata (Ex.: fagócitos, proteínas solúveis, como: citocinas, do sistema complemento e barreiras epiteliais) e adaptativa (Ex.: anticorpos e células efetoras citotóxicas). A imunidade inata, como primeira linha de defesa, não desencadeia

memória imunológica, entretanto, é responsável pela ativação da resposta imune adaptativa. Esta primeira linha de defesa, não-específica, é útil na proteção contra microrganismos piogênicos (Ex.: *Staphylococcus aureus*), fungos (Ex.: *Candida albicans*) e parasitas multicelulares (Ex.: *Ascaris lumbricoides*, nematelminto) (Figura 10) (PEAKMAN & VERGANI, 1999).

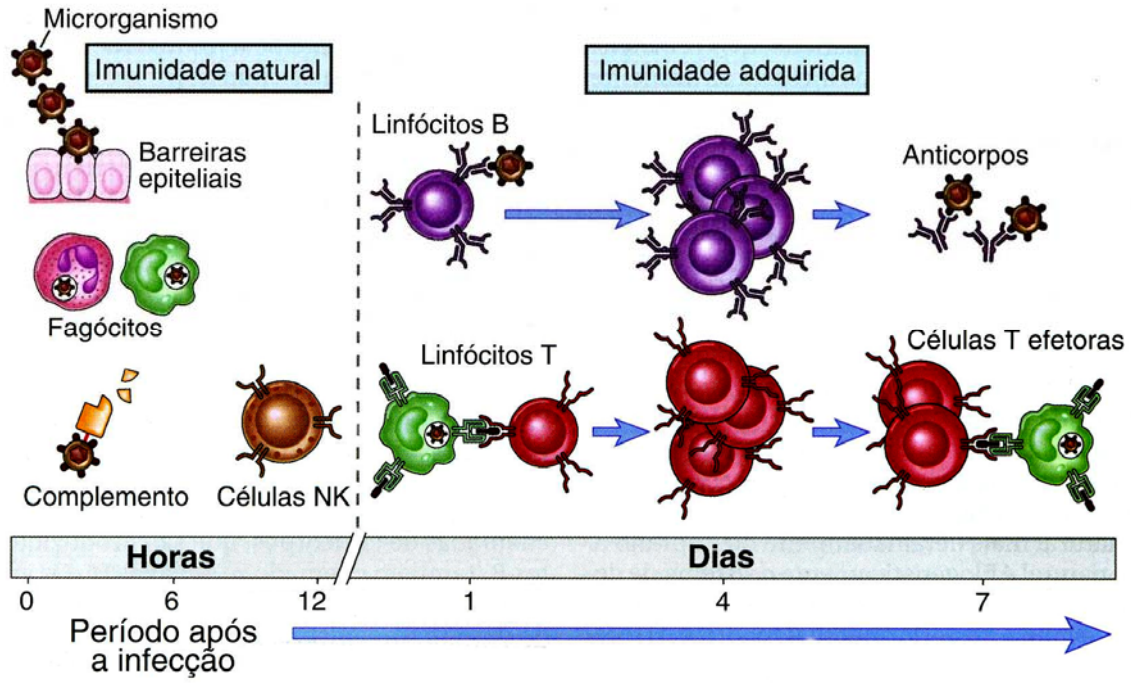

Figura 10. Esquema das imunidades natural e adquirida. Os mecanismos da imunidade natural fornecem a defesa inicial contra infecções. As respostas imunológicas adquiridas se desenvolvem posteriormente e consistem na ativação dos linfócitos. A cinética das respostas imunológicas natural e adquirida é um estimativa e pode variar em diferentes infecções, demonstrando o período de resposta das imunidades natural e adquirida, frente ao patógeno e os tipos ativados no processo. Fonte: (ABBAS & LICHTAMN, 2008).

Enquanto que, a segunda linha de defesa, específica, desencadeia ações letais frente aos antígenos “não-próprios”, assim resultando na produção de células de vida longa e com capacidade para memória. A resposta imune humoral, mediada por anticorpos, age diante de partículas virais livres de fluidos corporais e podem prevenir a

reinfecção viral, enquanto que a resposta imune celular é essencial para a remoção de células infectadas por vírus e pela geração de memória imunológica (Figura 11) (STANLEY, 2005).

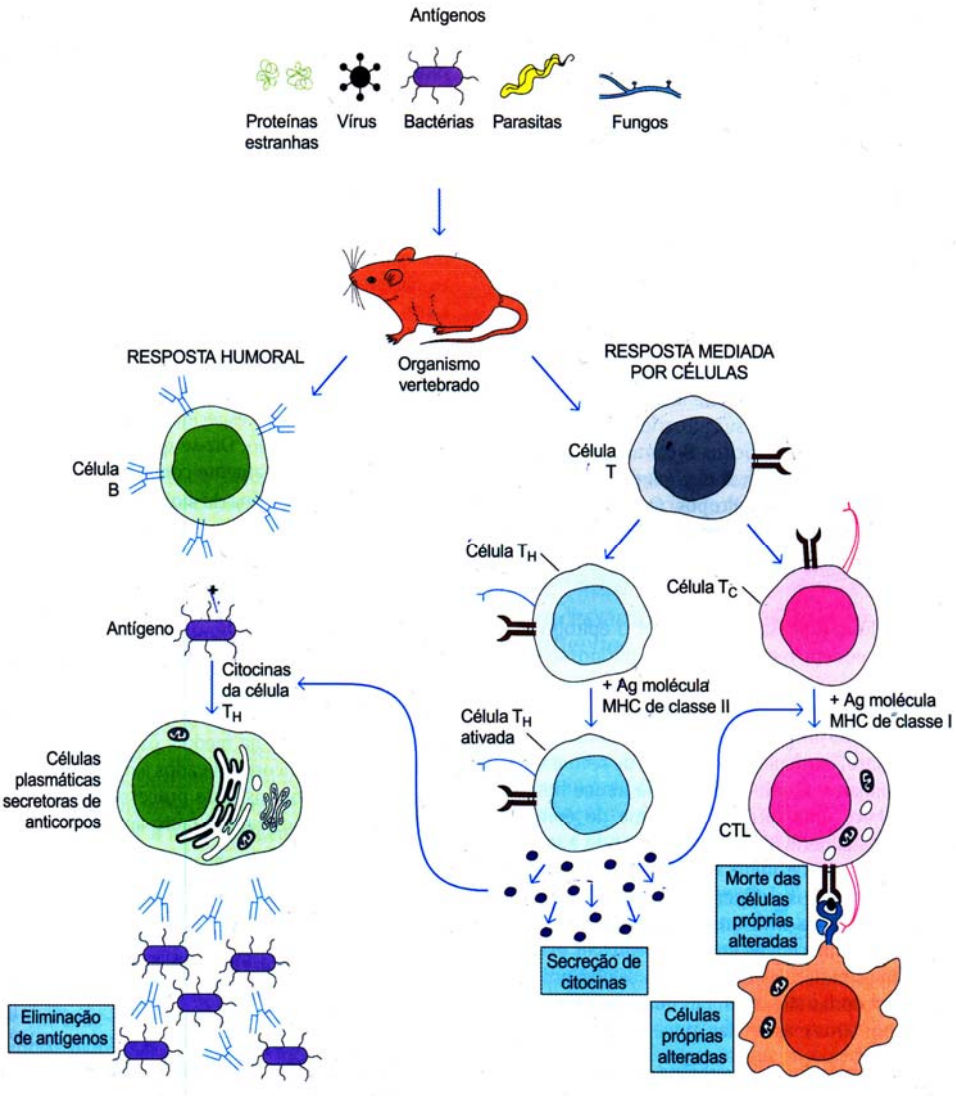

Figura 11. Visão geral dos ramos humoral e mediada por células do sistema imune. Na resposta humoral, as células B interagem com o antígeno (Ag) e, então, diferenciam-se em células plasmáticas secretoras de anticorpos (Ac). O anticorpo secretado se liga ao antígeno e facilita a sua eliminação do organismo. Na resposta mediada por células, várias subpopulações de células T reconhecem os antígenos apresentados nas próprias células. As células LT_H respondem aos antígenos através da produção de citocinas. As células TCs respondem aos antígenos pelo desenvolvimento de linfócitos T citotóxicos (LTCs) que medeiam a morte das células próprias alteradas (Ex.: células infectadas por vírus) (GOLDSBY *et al.*, 2002).

2.6.1 Sistema Imunológico × HPV

Os vírus por serem microrganismos intracelulares obrigatórios, que se replicam no interior da célula, freqüentemente utilizam ácidos nucléicos e o "maquinário" da síntese de proteínas do hospedeiro. Os principais mecanismos de imunidade inata contra os vírus são a inibição da infecção pela ação dos interferons (IFNs) tipo I e a morte das células infectadas mediada pela célula NK (*Natural Killer*). Enquanto que, a imunidade adaptativa contra infecções virais é mediada por anticorpos, os quais bloqueiam a ligação do vírus e a entrada nas células do hospedeiro, e por linfócitos "T" citotóxicos (LTCs), os quais eliminam a infecção pela destruição das células infectadas (ABBAS & LICHTMAN, 2008).

As infecções causadas pelo HPV, caracterizadas pela estreita especificidade das espécies e pelo tropismo tecidual, infectam queratinócitos basais primitivos (provavelmente, célula tronco), via microabrasão na mucosa do epitélio, todavia expressam elevados níveis de proteínas virais em camadas superiores do epitélio escamoso. Evidências clínicas indicam que a expressão gênica viral está relacionada ao queratinócito ou às células com o potencial de maturação escamosa (STERLING *et al*., 1993; MIDDLETON *et al*., 2003).

O epitélio é uma interface entre os vírus e o ambiente externo, por um lado, e o SI, por outro lado. Os efeitos da barreira imune inata são reforçados por elementos, tais como: bacilo *Doderlein*, acidez vaginal, secreção e defensinas (peptídios com atividade bactericida e viricida) por células epiteliais, além da drenagem através da mucosa cérvico-vaginal. A superfície do corpo provém uma barreira aos insultos mecânicos e

microbianos. Esta barreira microbiana pode ser, especialmente, significativa na mucosa da cavidade oral e em outras mucosas, onde o potencial para infecções bacterianas e fúngicas é considerável. Estes grupos de fatores na presença de uma infecção viral ativam a respostas imune, adaptativa. As razões para a enorme diversidade dos tipos de vírus, assim como para a restrição de sua propagação para a pele ou sítios internos da mucosa, ainda, são desconhecidos (MIDDLETON *et al*., 2003).

O período entre a infecção e o aparecimento de lesões é consideravelmente variável, sugerindo que o vírus pode efetivamente se esquivar do SI, pois a infecção causada pelo HPV, além de não ser acompanhada por uma inflamação, não reflete um "sinal perigoso" visível e capaz de alertar esse sistema. Dessa forma, a infecção crônica é resultante de uma não observação, pelo SI do hospedeiro, frente à permanência prolongada do patógeno (STANLEY, 2005).

O modelo básico da resposta imune inata, não-específica e sem memória imunológica, diante do HPV envolve uma ação imunomoduladora das citocinas e efetores celulares, tais como: monócitos, macrófagos, células *NKs* e células apresentadoras de antígenos (APCs, *antigens presented cells*). No processamento e a apresentação dos antígenos virais pelas células de Langerhans (CLs), um grupo particular de receptores de reconhecimento padrão (PRRs, *pattern recognition receptors*), que reconhecem padrões moleculares associados aos patógenos (PAMPs, *pathogens-associated molecular pattern*), presentes na estrutura do HPV, é reconhecido. Juntamente com esse reconhecimento, entre PRR e PAMP, a ligação de citocinas pró-inflamatórias às CLs parece ser de fundamental importância, pois ativa tanto o processamento do antígeno como também a sua migração para os linfonodos. Durante a migração, as CLs tornam-se maduras e altamente especializadas na

apresentação de antígenos via moléculas do complexo de histocompatibilidade principal de classe I (MHC, *major histocompatibility complex*) e capazes de ativar as células T virgens nos linfonodos fazendo com que eles, via eferente, migrem para o sítio de infecção, mas especificamente para os queratinócitos infectados como células T efetoras (GONÇALVES *et al*., 2004).

A resposta imune celular, específica, e com capacidade de gerar memória imunológica desempenha uma importante função no controle de neoplasias associadas à infecção pelo HPV. Geralmente, lesões relacionadas a essa infecção ocorrem em populações com imunidade celular deficiente (Ex.: pacientes submetidos à terapia imunosupressora, em decorrência de transplante de órgãos). Além disso, as lesões desaparecem, freqüentemente, quando a imunosupressão é eliminada e/ou reduzida (WU, 1994).

Estudos histológicos têm demonstrado a regressão espontânea de verrugas genitais, assim demonstrando indícios da natureza da resposta imune celular na infecção pelo HPV. Àquelas que não regrediram foram caracterizadas pela ausência de células do SI, no local da infecção, que por análise histológica das verrugas genitais, em regressão, um considerável infiltrado de células T, CD4+ e CD8+, e macrófagos foi revelado no estroma e epitélio. Esse infiltrado expressa marcadores de ativação, tais como citocinas pró-inflamatórias, IL-12, TNF-α e IFN-γ, além da regulação da adesão das moléculas requeridas pelo tráfego de linfócitos no endotélio dos capilares das verrugas. A IL-12 bioativa é expressa não apenas pelas células dendríticas (CDs) e macrófagos, nesse processo regressivo, mas como também em queratinócitos infectados (STANLEY, 2005).

Complementando as respostas imunes celular e humoral do hospedeiro, frente ao HPV, outros fatores imunológicos também desempenham funções importantes em defesa da infecção causada por esse vírus, tais como: quantitativo das APCs; alterações na secreção de citocinas, assim como mudança na expressão das MHCs. As MHCs são muito importantes na regulação da resposta imune na cérvix, pois antígenos são apresentados por MHC de classe II em 50% das lesões intraepiteliais de alto grau, enquanto que a expressão de antígenos por MHC de classe I é extremamente reduzida ou ausente em mais de 75% das lesões intraepiteliais cervicais e em carcinomas cervicais (CROMME *et al*., 1993).

O HPV se esquiva, eficientemente, da resposta imune inata e retarda a resposta imune adaptativa. Existem várias razões para esses processos acontecerem, como exemplo, a replicação e liberação do HPV não causam morte celular, pré-requisito para a inflamação, assim não demonstrando um "sinal perigoso" para o SI. A liberação de citocinas pró-inflamatórias são importantes para a ativação e migração de células dendríticas para o local atingido, entretanto, em casos de infecção por HPV, no epitélio escamoso, essa liberação é escassa ou ausente (KUPPER *et al*., 2004).

2.6.2 Sistema Imunológico × *C. trachomatis*

A infecção sexualmente transmitida causada por *C. trachomatis***,** considerada como um sério problema de saúde pública, tem sido alvo de programas de controle assistenciais, em países em desenvolvimento. As conseqüências adversas, em longo prazo, oriundas da infecção por *C. trachomatis* são doença pélvica inflamatória (PID, *pelvic inflammatory disease*), tal como a infertilidade e gravidez ectópica (EP, *ectopic*

pregnancy), com intensa dor pélvica, as quais levantaram um questionamento: “Qual a hipótese da maior parte das infecções, causadas por *C. trachomatis*, serem assintomáticas e capazes de desencadear manifestações tão graves como cervicite, endometrite, PID, EP e abcesso na glândula de Bartholin, nas mulheres?” (FERREIRA & ÁVILA, 2001).

A imunidade diante de infecções bacterianas é extremamente complexa, devido aos diversos fatores de patogenicidade utilizados pelas bactérias para aumentar a sua sobrevida. Com efeito, um grande número, senão a maioria dos fatores de virulência das bactérias, destina-se a evitar ou neutralizar as defesas do hospedeiro. A defesa primária inespecífica frente infecções bacterianas é proporcionada pelos granulócitos, que ingerem e matam a maioria dos patógenos potenciais. A lectina ligadora de manose (MBL, *Mannose Binding Lectin)* é uma proteína sérica da resposta imune inata capaz de ativar o sistema complemento e de se ligar ao carboidrato manose, em diferentes microrganismos. Em relação à imunidade específica, a mesma é necessária para proteger o hospedeiro diante das bactérias encapsuladas ou intracelulares, promovendo o desenvolvimento de anticorpos, que podem aumentar o processo de destruição através de sua atividade opsônica ou de fixação de complemento ou da imunidade mediada por células T, capaz de ativar a atividade microbicida dos macrófagos (PARSLOW *et al*., 2004).

No estudo da imunidade inata ou *in natura* de seus componentes, à medida que se sabe a respeito dos processos fisiológicos, bem como dos patológicos, têm-se alternativas e possibilidades para o conhecimento detalhado do funcionamento desse tipo de imunidade frente aos patógenos. Em especial, o conhecimento do verdadeiro papel da MBL de encontro às doenças desencadeadas pelos microrganismos, em

destaque as infecções provocadas pela bactéria *C. trachomatis*, revela uma nova possibilidade de cura, uma vez que, a MBL leva a uma rota para ativação do sistema complemento, auxiliando, portanto, a debelar os invasores estranhos (SAEVARSDOTTR *et al*., 2004). Além disso, a MBL é um importante constituinte do sistema imunológico inato, sendo também uma das proteínas presentes no sistema complemento, onde participa da ativação através da terceira via (FUJITA, 2002). Segundo Jack e colaboradores (2001), é relevante comentar o aumento significativo nas evidências sobre a associação da MBL com doenças humanas, onde a proteína teria um papel principal na modulação dos processos inflamatórios.

Recentes progressos na elucidação da imunobiologia da infecção por *C. muridarum*, em camundongos, têm auxiliado nas interpretações imunológicas dos estudos realizados na infecção humana por *C. trachomatis,* assim proporcionando o desenvolvimento de um modelo comum da imunidade. Nessa realidade, mecanismos de evasão da *C. trachomatis* puderam ser elucidados, tais como: sobrevida aumentada nos meios intra e extra-celulares do hospedeiro; resposta inflamatória reduzida; resposta imune inata reduzida e habilidade de persistência como formas alternativas intracelulares (BRUNHAM & LADINO, 2005).

Uma característica das bactérias intracelulares facultativas é sua capacidade de sobreviver e mesmo de se replicar dentro dos fagócitos. Como esses microrganismos são capazes de encontrar um local onde estejam inacessíveis aos anticorpos circulantes, sua eliminação requer os mecanismos da imunidade mediada por células. As conseqüências patológicas da infecção por muitas bactérias intracelulares são devidas à resposta do hospedeiro a esses microrganismos (ABBAS & LICHTMAN, 2008).

O epitélio da pele e tecidos de mucosa funciona como um mecanismo de barreira para a invasão de patógenos microbianos. Evidenciou-se, há duas décadas, que as células epiteliais são a maior fonte de peptídeos antimicrobianos capazes de desempenhar uma importante defesa local no hospedeiro. Estudos na sua estrutura, origem, expressão e ações também têm sido revelados e correlacionados com suas atividades imunológicas, estas consideradas antimicrobianas naturais, em suas funções (GANZ, 2003).

As células epiteliais são os alvos principais, durante a infecção por *C. trachomatis*, sendo os receptores celulares responsáveis pela entrada desse patógeno, ainda não são bem esclarecidos; entretanto, no caso do sorotipo L2, a sua internalização é iniciada pelo ataque da bactéria em glicosaminoglicanos, heparan sulfatos, na superfície celular. Essas células epiteliais presentes em várias superfícies de mucosa, ao longo do corpo, atuam tanto como uma barreira de defesa na entrada de patógenos, quanto como alvos celulares iniciais para a infecção microbiana. As mesmas servem como indicadores sensíveis às infecções pelo início de ativação da resposta imune precoce do hospedeiro, através da secreção de quimiocinas e citocinas pró-inflamatórias. Citocinas, tais como, IL-1α, IL-1β, IL-6, TNF-α e GM-CSF produzidas pelas células epiteliais recrutam as células do sistema imune para a superfície epitelial/mucosal para auxílio da ativação de macrófagos e CDs e na diferenciação de linfócitos efetores (STADNYK, 1994). Além dessas, a IL-18 juntamente com a IL-12 regulam as respostas das citocinas da Th1, expressas em células epiteliais intestinais, importantes na diferenciação de células (PIZARRO *et al*., 1999).

O padrão de produção *in vivo* de citocinas é fortemente correlacionado com a progressão e/ou resolução de doenças infecciosas severas, incluindo as ISTs. A genética do hospedeiro é considerada como um dos principais fatores que regulam a diferenciação e polarização para uma resposta imune Th1 e Th2, estando relacionada com a duração da infectividade da IST. Dentre os principais fatores genéticos podem ser citados o polimorfismo na região promotora da MHC e diferenças na regulação das citocinas, como exemplo, a secreção de INF-γ pelas células Th1 limita a infecção por *C. trachomatis*, pela depleção do ferro e triptofano intracelular. Dessa forma, fatores genéticos que influenciam níveis de secreção de INF-γ, são condições influenciadoras tanto na duração da infecção e na infectividade da doença clamidial. Além disso, alguns estudos têm relatado a relação entre diferentes genes de MHC e a susceptibilidade à infecção por *C. trachomatis,* como exemplo, utilizando genes MHC de camundongos Knockout, Morrisonn e colaboradores (1995) demonstraram que MHC de classe II é importante na defesa do hospedeiro frente à infecção por *C. trachomatis*, no trato genital (BEATTY *et al*., 1993; YANG *et al*., 2006).

As células epiteliais e circulantes, do sistema imune inato, possuem PRRs. As duas famílias mais importantes, desses PRR, são as proteínas TLR (TLR, *toll-like receptor*) e NOD (NOD, *nucleotide-binding oligomerization domain*). Nas infecções causadas por *C. trachomatis*, no trato genital feminino, os PRRs da resposta imune inata estão envolvidos na remoção da infecção. Variações genéticas nos PRRs podem contribuir na persistência e/ou no aumento do risco na patologia tubária, pois um elemento chave na resposta imune normal à infecção por *C. trachomatis* é o adequado

reconhecimento do patógeno, pelos PRRs, nas células epiteliais do trato genital (HARTOG *et al*., 2006).

É plausível a importante participação dos TLRs no mecanismo de defesa do hospedeiro frente às infecções genitais causadas pela *C. trachomatis*, pelo fato desses receptores serem capazes de reconhecer os PAMPs da *C. trachomatis* e serem expressos nas células epiteliais no trato genital humano. TLR2 é o PRR para o componente peptidoglicano da *C. trachomatis*, enquanto que o TRL4 é o PRR para os componentes lipopolissacarídeos e proteína de choque (*hsp, heat shock protein*). TLR1, TLR3, TLR5 e TLR6 também estão presentes no trato genital feminino humano, entretanto não são reconhecidos pelos PAMP da *C. trachomatis* (PIOLI *et al*., 2004). Além disso, em decorrência da *C. trachomatis* ser um patógeno intracelular e possuir lipopolissacarídeos e peptidoglicanos, uma função dos NODs intracelulares no reconhecimento da *C. trachomatis* tem sido sugerida (DERBIGNY *et al*., 2005; WELTER-STAHL *et al*., 2006).

Estratégias relevantes de evasão da *C. trachomatis* baseiam-se na biossíntese de triptofano para escapar do mecanismo de defesa do interferon-γ (IFN-γ) e variação alélica da sua proteína de superfície dominante - proteína principal de superfície de membrana (MOMP, *major outer-membrane protein*). Pelo fato da MOMP ser considerada como um importante alvo da imunidade, a especificidade da imunidade de diferentes sorotipos da *C. trachomatis* pôde ser estudada, no modelo *C. muridarum*, em camundongos (BRUNHAM, 1999). Além disso, sabendo-se que a *C. trachomatis* é uma importante causa de danos imune-mediados no trato reprodutivo de pacientes infectadas, antígenos clamidiais e fatores genéticos do hospedeiro têm sido

identificados como contribuintes em eventos imunopatológicos, entretanto, uma maior compreensão dos componentes específicos envolvidos na destruição *versus* resposta imune protetora às infecções por *C. trachomatis*, ainda está longe de ser bem elucidada (RAULSTON *et al*., 2007).

2.6.3 Vacinas

Por décadas a genética e a maquinaria celular dos microrganismos foram manipuladas para permitir a produção de proteínas recombinantes ou heterólogas, hormônios humanos, antígenos, peptídeos, enzimas e outras moléculas que têm potencial uso clínico ou industrial e são, freqüentemente, encontradas em baixos níveis na natureza ou são particularmente difíceis de purificar. A obtenção rápida de proteína recombinante é necessária para a produção de novas moléculas terapêuticas, como vacinas antigênicas, IFN-γ, IL-7, fator de crescimento insulina-dependente, leptina humana, dentre outras (CHAVES *et al*., 1999; CHOI *et al*, 2005).

As vacinas de HPV, preventivas e terapêuticas prometem e podem tornar-se uma esperança poderosa para o controle da incidência do câncer cervical, assim como da mortalidade e morbidade. Após a associação da gênese do câncer cervical com o HPV e a influência do SI, a idéia de se desenvolver vacinas frente a essa infecção viral tornou-se promissora (RODEN *et al*., 2004).

Associações entre anticorpos frente às proteínas do HPV e doenças relacionadas ao vírus foram estabelecidas por diversos estudos na literatura. A maioria baseia-se em antígenos sintéticos ou recombinantes que se assemelham às proteínas

autênticas de expressão tardia (não-estruturais) e precoce (estruturais) do HPV, na função e imunogenicidade, tendo sido detectados por diversos experimentos, incluindo ELISA, *Western blotting* e, mais recentemente, a imunoprecipitação. Dentre os antígenos utilizados nesses estudos, proteínas recombinantes de expressão tardia do HPV, componentes do capsídeo viral, denominadas de partículas vírus-semelhantes (VLPs), têm sido as mais documentadas (PINTO *et al*., 2002).

A capacidade de produzir em larga escala as VLPs-HPV resultou em ensaios clínicos de fase II promissores na prevenção de mulheres jovens, sexualmente ativas, da infecção pelos tipos oncogênicos do HPV. A vacina desenvolvida pela Merck® contém derivados de dois tipos de HPV oncogênico mais comum, 16 e 18, bem como VLPs derivadas de dois tipos comuns verrugas genitais, 6 e 11. Esta foi testada em vários ensaios de fase II, principalmente em mulheres jovens, em sua adolescência ou no início de seus vinte anos. Nestes estudos, VLPs derivadas de cepas oncogênicas de HPV foram administradas por injeção intramuscular (KIRNBAUER *et al*., 1993).

A GlaxoSmithKline® também produziu, em larga escala, as VLP-HPV, 16 e 18, em células de *Spodoptera frugiperda* SF9 com o adjuvante AS04, em mistura com hidróxido de alumínio e 3 lipídio A monofosforil deacetilado, para a realização de ensaios clínicos de fase II. Níveis muito elevados de proteção frente à infecção pelos tipos de HPV 16 e 18, assim como anormalidades histológicas cervicais (93%) foram observadas em mulheres jovens até 27 meses após a vacinação (HARPER *et al*., 2004).

Recentemente, agências de regulamentação de medicamentos de vários países, como a *Food and Drug Administration* (FDA) dos Estados Unidos e a Agência Nacional

de Vigilância Sanitária (ANVISA/MS) do Brasil, aprovaram para comercialização a primeira vacina desenvolvida para a prevenção das infecções mais comuns que causam a condilomatose genital (HPV 6 e 11) e o câncer do colo do útero (HPV 16 e 18). A incorporação da vacina contra HPV pode se constituir, no futuro, em importante ferramenta no controle do câncer do colo do útero (INCA, 2008).

Em relação às estratégias vacinais para *C. trachomatis* MOMP e OMP2 têm sido adotadas como antígenos imunodominantes. A OMP2 possui uma seqüência de aminoácidos, altamente conservada, dentre os diferentes sorotipos da *C. trachomatis*, em relação à MOMP, além de conter epítopos celulares para TCD4 + e TCD8 +, assim, em uma vacina, a OMP2 pode fornecer uma proteção contra os vários sorotipos desse patógeno. Experiências recentes têm demonstrado que a inclusão de OMP2 melhora consideravelmente a proteção potencial das vacinas à base de MOMP. No entanto, se estes antígenos de células T fornecem uma proteção imunológica efetiva frente à *C. trachomatis*, necessitam ser determinadas (STARNBACH *et al*., 2003; BRUNHAM & LADINO, 2005; EKO *et al*., 2007).

2.7 Lectina Ligadora da Manose (MBL)

O estudo de lectinas iniciou-se em 1888 quando um jovem doutor, Peter Hermann Stillmark, da Universidade Dorpat (agora Universidade de Tartu na Estônia), apresentou sua tese de Doutorado intitulada "*Ueber Ricin, ein giftiges Ferment aus den Samen Von Ricinus comm. L. und einigen anderen Euphorbiaceen*" a qual se referia a descoberta de uma proteína tóxica, denominada Ricina, presente no feijão castor que tinha a propriedade de aglutinar células do sangue ampliando desta forma o estudo de

aplicações destas proteínas (MACIEL DE CARVALHO, 2008). Este evento é internacionalmente conhecido como o nascimento de um novo ramo na ciência chamado de lectinologia (KENNEDY *et al*., 1995; GABOR *et al*., 2001; BIES *et al*., 2004).

Lectinas são ubíquas na natureza e, devido à sua única propriedade de se ligar aos carboidratos presentes na superfície celular, desempenham um importante papel em eventos celulares como aglutinação, reconhecimento celular, simbiose, estimulação da proliferação, opsonização, metástase e apoptose (DUTTA *et al*., 2005). Desempenham um significante papel na resposta imune do hospedeiro conjugando-se especificamente às moléculas de carboidratos expressas sobre patógenos, agindo como opsoninas e estimulando sua destruição por macrófagos (RICHARDS *et al*., 2003). Além disso, essas proteínas ainda podem ser classificadas, de acordo com o carboidrato específico a que preferencialmente se ligam, através de pontes de hidrogênio, coordenações metálicas, interações de van der Walls e interações hidrofóbicas (Tabela 7) (NOMURA *et al*., 1998; PEUMANS e VAN DAMME, 1997).

As lectinas podem ser usadas como ferramentas para estimular a proliferação de linfócitos para tipagem de grupos sanguíneos, para detectar componentes de carboidratos presentes em superfícies de células normais e cancerígenas. Estas proteínas podem ser utilizadas como ferramenta para a produção dos chamados medicamentos-inteligentes, no qual diferem dos tradicionais, por atuarem em células específicas do organismo evitando efeitos colaterais, do tipo provocado pela quimioterapia (BELTRÃO *et al*., 2003).

Tabela 7: Classificação das lectinas de plantas nos grupos de reconhecimento a carboidrato. Modificado (PEUMANS e VAN DAMME, 1997). Adaptado por Maciel de Carvalho, 2008.

GRUPO	ESPECIFICIDADE	LECTINA (FONTE)
Fucose	Fucose	*Ulex europaeus* aglutinina I (Tojo)
N-acetilglicosamina	GlcNAc	*Triticum aestivum* (Germe de trigo)
Galactose/N-acetilgalactosamina	Galactose>>GalNAc Gal= GalNAc Gal<< GalNAc	*Artocarpus integrifolia* (Jacalina) *Clerodendron trichotomum* (*Harlequin glorybower* ou *Clerodendro japonês*) *Glycine max* (Feijão de soja)
Manose	Manose/Glicose Manose/ Maltose Manose	*Canavalia ensiformis* (Concanavalina A) *Calystegia sepium* (Bons dias) *Galanthus nivalis* (Campanhia branca)
Complexo Glicano	Glicoproteína	*Phaseolus vulgaris* Aglutinina (PHA)

A MBL, uma proteína sérica, incluída entre as famílias das colectinas, é constituída por um polipeptídeo dividido em quatro domínios distintos: (1) um pequeno domínio rico em cisteína, (2) seguido por um domínio de colágeno, (3) uma porção α-hélice chamada de pescoço (*neck*) e (4) um domínio reconhecedor de carboidratos (DRC). A subunidade desta proteína é formada quando três polipeptídios se associam através das pontes dissulfetos formadas entre os resíduos de cisteína do domínio rico desse aminoácido (WALLIS, 2003) (Figura 12).

No homem, existem duas outras colectinas, chamadas de SP-A (Proteína Surfactante A, *Surfactant Protein-A*) e SP-D (Proteína Surfactante D, *Surfactant Protein-D*). Todas as três proteínas são macromoléculas e ambas, MBL e SP-A, têm estrutura similar ao componente C1q do sistema complemento. Contudo, no caso da MBL, vários arranjos oligoméricos de dímeros a hexâmeros têm sido reportados. Esses oligômeros

são baseados em subunidades compreendidas em três peptídeos idênticos de 32 KDa. Cada cadeia é caracterizada por um domínio de lectina, uma região hidrofóbica, uma região colagenosa e uma região rica em cistina e N-terminal. A região colagenosa interage para conferir a tripla hélice característica. Na porção terminal, cada cadeia adota uma conformação de enovelamento, o que favorece para o aparecimento da característica de uma proteína globular. Cada domínio ainda é formado por íons cálcio, N-acetil-D-glicosamina, manose, N-acetil-galactosamina, ficose e glicose (TURNER *et al*., 2003).

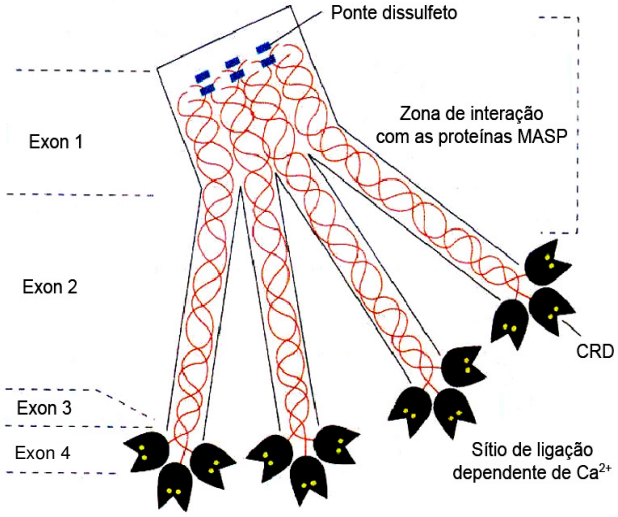

Figura 12. Desenho esquemático da estrutura da MBL e suas regiões: domínio de reconhecimento de carboidratos (DRC), as regiões dos quatro éxons, sítios de ligação dependentes de cálcio, zona de interação com as proteínas MASP e as pontes dissulfetos. Modificado (TURNER *et al*., 1996).

A MBL sérica é uma estrutura multimérica composta de mais de 6 trímeros (complexo multimérico reunidos como um *bouquet* de tulipas) que circulam na corrente sangüínea como tetrâmeros, pentâmeros ou hexâmeros da unidade estrutural (WHORTLEY *et al.,* 2005) e cada peptídeo possui um domínio C terminal dependente

de cálcio que reconhece oligossacarídios ricos em manose e N-acetilglicosamina presentes em uma ampla variedade de bactérias, vírus, fungos e parasitas (TURNER *et al.*, 1996).

A MBL é um importante componente do sistema imune inato e uma das mais de 30 proteínas do sistema complemento. A mesma ativa o sistema complemento por uma rota adicional, sendo o principal componente da imunidade inata. Há evidências de que a proteína tem pelo menos quatro funções distintas: (i) ativação do sistema complemento; (ii) a promoção da opsonização e fagocitose complemento-independente; (iii) a modulação da inflamação e (iv) a promoção da apoptose. De todas essas funções, a ativação do complemento tem sido estudada mais extensivamente, uma vez que os mecanismos MBL-mediados (Ex.: opsonização e fagocitose) não são muito bem esclarecidos. A importância biológica dessa terceira rota de ativação do complemento é relevante em conseqüências clínicas observadas em casos de sua deficiência (FUJITA, 2002).

A via da lectina é considerada similar à via clássica do complemento, entretanto é ativada na ausência de anticorpo pela ligação de polissacarídios microbianos a lectinas circulantes, tais como a MBL. A MBL se liga aos resíduos de carboidratos da superfície dos microrganismos e como é estruturalmente similar ao C1q, desencadeia o sistema complemento ou por ativação do complexo enzimático C1r–C1s ou por associação a serinoproteinases, MASP-1 e MASP-2. A MASP-1 e MASP-2 são semelhantes aos C1r e C1s do sistema complemento, respectivamente, enquanto que a MBL é semelhante ao C1q do mesmo. A formação do complexo trimolecular MBL/MASP-1/MASP-2 resulta na clivagem C4 e C2 para formar C4b2a (C3

convertase). A proteína central do sistema complemento C3 é clivada e seu fragmento maior C3b é depositado na superfície microbiana em que o complemento é ativado. O C3b se torna covalentemente ligado aos microrganismos e atua como uma opsonina para promover a fagocitose dos mesmos. Um fragmento menor, C3a, é liberado e estimula a inflamação agindo como um quimioatraente para neutrófilos. O C3b se liga a outras proteínas do complemento para formar uma protease que cliva uma proteína chamada C5, gerando um peptídio secretado (C5a) e um fragmento maior (C5b) que permanece fixado às membranas celulares microbianas. O C5a estimula a entrada de neutrófilos no local da infecção, bem como o componente vascular da inflamação aguda. O C5b inicia a formação de um complexo de proteínas C6, C7, C8 e C9, as quais são reunidas em um poro de membrana que causa a lise das células nas quais o complemento está ativado. Na via da lectina fora o fato se ser ativada na ausência de anticorpo, as demais etapas desta via são as mesmas da via clássica (Figura 13) (BENJAMINI *et al*., 2002).

A deficiência da MBL tem sido relacionada a um grande número de doenças: infecções recorrentes, principalmente em crianças, imunodeficiências primárias e secundárias, tais como, infecção pelo HIV, terapia para câncer, doenças da artéria coronária/ aterosclerose, fibrose cística, doenças autoimunes (Ex.: lupus eritematoso e artrite reumatóide) (SUMMERFIELD *et al*., 1997; CHRISTIANSEN *et al*., 1999; PETERSLUND *et al*., 2001).

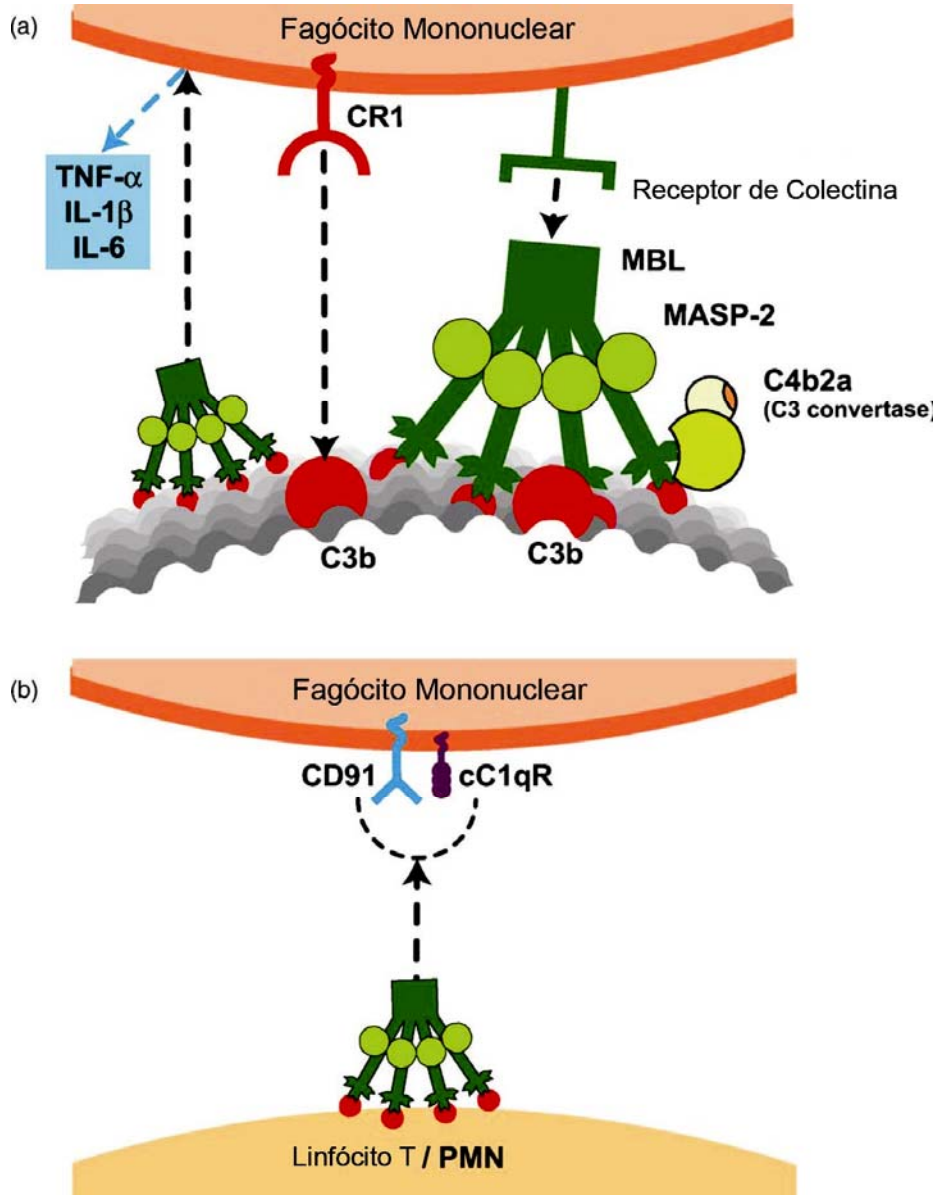

Figura 13: Funções da MBL. a) A MBL liga-se a superfície dos microrganismos e promove a opsofagocitose por 2 mecanismos. O mais importante é ativação do sistema complemento pela via da lectina mediada pela serino protease MASP-2 capaz de formar C3 convertase C4b2a. Esta é responsável pela clivagem de C3 e geração de múltiplos fragmentos de C3, os quais se ligam covalentemente à superfície do microrganismo. Cada fragmento é reconhecido pelo receptor CR1, no fagócito. Alguns C3b são convertidos em iC3b e reconhecidos pelo CR3 receptor. A captura direta da MBL pelo fagócito tem sido proposta em um número de estudos, mas o receptor de colectina envolvido, ainda não tem sido identificado. A MBL é também hábil para promover a inflamação pela modulação dose-dependente da liberação de citocinas do monócito. b) Uma função da MBL em apoptose também tem sido proposta. As colectinas também têm demonstrado se ligar às células T apoptóticas e neutrófilos polimorfonucleares, pela região CRD. Subseqüentemente a captura pelos fagócitos mononucleares é requerida pelo reconhecimento da região colagenosa pelo cC1qR em associação com CD91. Modificado (TURNER, 1996).

O gene da MBL humana é polimórfico, situado no cromossomo 10 (q21-24) e codifica um produto protéico chamado de MBL-2 que compreende 4 éxons e 3 íntrons. O éxon 1 codifica o peptídeo sinal, a região N-terminal rica em cisteína e parte da região colagenosa, enquanto o éxon 2 codifica o restante da região colagenosa. O éxon 3 codifica a região hidrofóbica espiralada conhecida como pescoço e o éxon 4 o domínio de reconhecimento de carboidratos (DRC)(TURNER, 2003; TURNER, 2006)

Os sítios polimórficos da região promotora estão nas posições -550, -221 e +4 do gene da MBL e representam os *loci* H/L, X/Y e P/Q respectivamente. Estes três *loci* estão intimamente ligados e devido ao desequilíbrio da ligação, apenas sete haplótipos (HYPA, LYQA, LYPA, LXPA, LYPB, LYQC e HYPD) são comumente encontrados. Destes, o haplótipo HYP está associado com concentrações plasmáticas normais e altas de MBL, enquanto concentrações baixas são freqüentemente associadas ao haplótipo LXP (MADSEN *et al*., 1998).

Em geral, o significado funcional do polimorfismo tem maior relevância quando se traduz em uma troca de um aminoácido no produto do gene ou quando diretamente afeta a transcrição, a estabilidade ou a tradução do RNAm. Ademais, existem evidências de que os polimorfismos localizados nos íntrons podem ter também significado funcional. Dessa forma, sendo a MBL uma proteína que se une às superfícies dos microrganismos e endotoxinas e promove a ativação do complemento e opsonizacão, a mesma pode sofrer ação dos polimorfismos. Assim, para sua máxima eficácia, esta proteína deve estar presente em valores plasmáticos adequados, que podem ser influenciados por um polimorfismo situado na região promotora do gene consistindo de troca de guanina por citosina na posição -550 *e* - 221 (CHICHE *et al*., 2000).

Recentemente, vários estudos em diferentes grupos étnicos têm mostrado a presença de mutações na região promotora e na seqüência codificadora do primeiro éxon nos códons 52 (Arginina→ Cisteína), 54 (Glicina→ Asparagina) e 57 (Glicina→ Glutamato), que são responsáveis por uma diminuição significativa no nível da proteína no soro (MULLIGHAN *et al*., 2000; STEFFENSEN *et al*., 2003).

Embora determinantes genéticos e ambientais de muitas doenças ainda estejam bem compreendidos, a identificação do nível de MBL em estágio recente da doença ajudará na identificação de pacientes que requerem um tratamento diferenciado. A relação entre a deficiência da MBL e uma ampla variedade de doenças têm levado a um aumento no interesse pela MBL. Há um crescente aumento pela genotipagem da MBL uma vez que a mesma influencia o fenótipo imunológico do indivíduo (MULLIGHAN *et al*., 2000).

Um recente estudo tem mostrado que a MBL (como C1q) pode ligar-se a células apoptóticas e iniciar seus processos pelos macrófagos (OGDEN, 2001). Esse processo apresenta requerimento de células com o receptor C1qR que se ligam com as regiões colagenosas da MBL. Uma grande variedade de afecções clínicas tem sido associada à deficiência de MBL, tais como a suscetibilidade aumentada para infecções bacterianas e virais (EISEN & MINCHINTON, 2003), a aterosclerose (RUGONFALVI-KISS, 2002), leucemias (SCHMIEDELOW, 2002), abortos (CHRISTIANSEN *et al*., 1999), outros patógenos externos e até infecções do trato respiratório (KOCH, 2001). Em relação aos fungos, sabe-se que a manose é um componente importante da parede celular dos mesmos e a MBL liga-se com alta avidez às diversas espécies, tais como: *Candida albicans, Aspergillus fumigatus*, bem como ao *Cryptococcus neoformans* (NETH *et al.,* 2000).

O papel da MBL nas doenças virais humanas está bem estabelecido a partir dos estudos com o HIV. Estes podem ser divididos em três diferentes áreas de investigação: *a)* o estudo da relação entre os níveis da MBL e a taxa e soroconversão; *b)* a relação entre níveis de MBL e a progressão da infecção pelo HIV e *c)* os efeitos da infecção pelo HIV nos níveis de MBL. Laboratorialmente, foi demonstrado, *in vitro*, que a infecção dos linfócitos CD4+ pelo HIV foi prejudicada pelas concentrações fisiológicas da proteína MBL. Em princípio, a insuficiência da MBL pôde aumentar a susceptibilidade ao vírus, após a exposição; afetar o curso da infecção pelo HIV; ou ter influência sobre infecções secundárias associadas à infecção pelo mesmo (GARRED & MADSEN, 1997).

A associação entre polimorfismos de um único nucleotídeo (SNPs, *single nucleotide polymorphisms*) da MBL-2 e susceptibilidade à infecção pelo HPV é possível apenas em relação aos genótipos funcionais. Entretanto, conforme dados da literatura, o polimorfismo individual da MBL-2 não está relacionado com o aumento ou diminuição da suscetibilidade à infecção pelo HPV. Na associação dos genótipos MBL-2 com o aumento da susceptibilidade à infecção pelo HPV deve-se considerar que o HPV é um vírus não-envelopado, assim dificultando a interação direta da MBL, em relação àqueles que possuem um envoltório. Dessa forma, por dados experimentais, a correlação significativa entre polimorfismo da MBL-2 aliado à infecção pelo HPV não foi observada (GUIMARÃES *et al*., 2008). Entretanto, VARDHANA e colaboradores (2004) demonstraram que a MBL participa na proteção contra infecções clamidiais, pois a variação alélica na expressão dessa proteína resulta em um decréscimo nos níveis de MBL, associado a um elevado risco de propagação dessas infecções.

2.8 Reação em Cadeia da Polimerase (PCR)

2.8.1 PCR Convencional

A técnica de PCR é um advento relativamente recente na história da biologia molecular, tendo sido desenvolvida nos anos 80 por Kary Mullis (Figura 14). Esta técnica é tão importante que em 1994 Mullis ganhou o prêmio Nobel. A idéia básica da técnica de PCR é bastante simples. Trata-se de uma metodologia *in vitro* que possibilita a reprodução de milhares de cópias de um determinado fragmento de DNA. Através desta técnica, uma seqüência particular de interesse pode ser amplificada, tornando-se majoritária na amostra de DNA. Deste modo, dois pequenos fragmentos de DNA (*primers*), normalmente de 20 pares de bases, são sintetizados *in vitro*. Estes *primers* são complementares às extremidades da região de DNA que se pretende amplificar (BROWN, 2003).

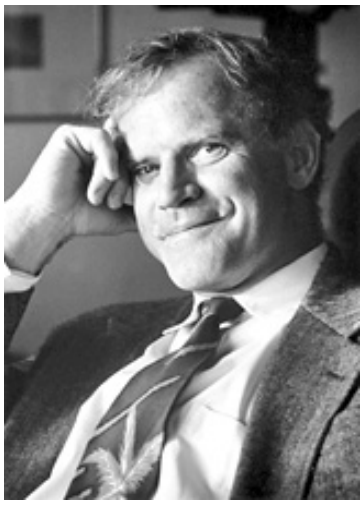

Figura 14. Foto do prêmio Nobel. Kary Mullis, responsável pelo desenvolvimento da técnica PCR.

http://nobelprize.org/nobel_prizes/chemistry/laureates/1993/mullis-autobio.html

A PCR permite a amplificação de segmentos curtos da molécula de DNA. Geralmente, uma reação de amplificação contém o DNA com a seqüência-alvo a ser amplificada, uma DNA polimerase termoestável, *Taq* polimerase, dois oligonucleotídeos iniciadores, desoxirribonucleotídeos (dNTPs), tampão de reação e concentração adequada de $MgCl_2$. O DNA com a seqüência-alvo, misturado com os componentes da reação, é colocado no termociclador, a fim do mesmo possibilitar os sucessivos ciclos de aquecimento e resfriamento rápido dessa amostra. Dessa forma, por exemplo, o aquecimento a 95º C possibilita a desnaturação do DNA-alvo, enquanto que a redução da temperatura para 45-65ºC possibilita o anelamento dos iniciadores e a 68-72ºC a extensão da cadeia de DNA (polimerização) é possibilitada. A amplificação do DNA-molde é obtida após a repetição, 20 a 30 ciclos, das etapas de desnaturação, anelamento e polimerização (Figura 15) (WALKER & RAPLEY, 1999*)*.

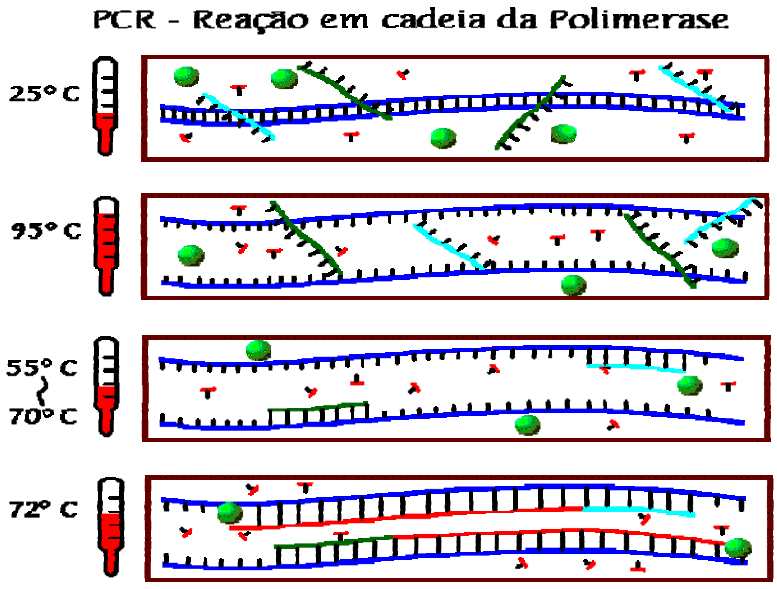

Figura 15. Desenho esquemático das etapas da PCR: desnaturação 94-96°C, anelamento ~65°C e polimerização a 72°C. http://en.wikipedia.org/wiki/Polymerase_chain_reaction.

Um dos frutos do projeto do genoma humano é a descoberta de milhões de seqüências de DNA variantes no genoma. A maioria destes variantes é de polimorfismos em um único nucleotídeo (SNPs), a qual possibilita o estudo da base genética de doenças complexas por populações semelhantes. Quatro métodos são comumente estudados para identificação de SNP (ou mutação): (i) identificação de polimorfismos por alteração de uma única fita, amplificado por PCR; (ii) análise de "heteroduplex"; (iii) sequenciamento direto e (iv) o recentemente descoberto detector de variantes "array" (VDA) (SOUZA, 2006).

2.8.2 PCR em Tempo Real

A PCR em Tempo Real é uma metodologia mais moderna que permite a quantificação dos produtos de amplificação gênica em todas as fases de uma reação de PCR (GINZINGER, 2002). Durante a PCR em Tempo Real, o acúmulo de produto amplificado (*amplicon)* é detectado em *"tempo real",* para cada ciclo da reação, por meio da excitação de fluorocromos que marcam sondas seqüência-específicas ou iniciadores (*primers*) utilizados na reação (FADERL *et al.,* 2004). O uso de transcrição reversa associada a PCR em Tempo Real tornou a quantificação de mRNA mais simples e precisa (BARBANY *et al.,* 2000). Essa PCR requer uma plataforma de instrumentação que contém um termociclador acoplado a um sistema óptico para a excitação da fluorescência e captura da emissão, além de um computador para aquisição de resultados e análise final da reação (NOVAIS & PIRES-ALVES, 2004).

Enquanto a reação de PCR se processa, uma polimerase sintetiza novas cadeias a partir dos *primers*, clivando a sonda correspondente, assim resultando no aumento de um sinal fluorescente que é captado a cada ciclo até atingir um limiar de detecção

(*threshold*), no qual todas as amostras podem ser comparadas. Este limiar corresponde ao momento utilizado para análise da fluorescência. É um ponto definido pelo pesquisador e obrigatoriamente deve estar na faixa em que a quantidade de fluorescência gerada pela amplificação das amostras torna-se significativamente maior do que a fluorescência de base (*background*). O limiar é definido na fase exponencial da reação de PCR, quando a quantidade de produto formada traduz, de forma satisfatória, a concentração inicial de fitas moldes (mRNA/cDNA) amplificadas pela reação (Figura 16) (GINZINGER, 2002).

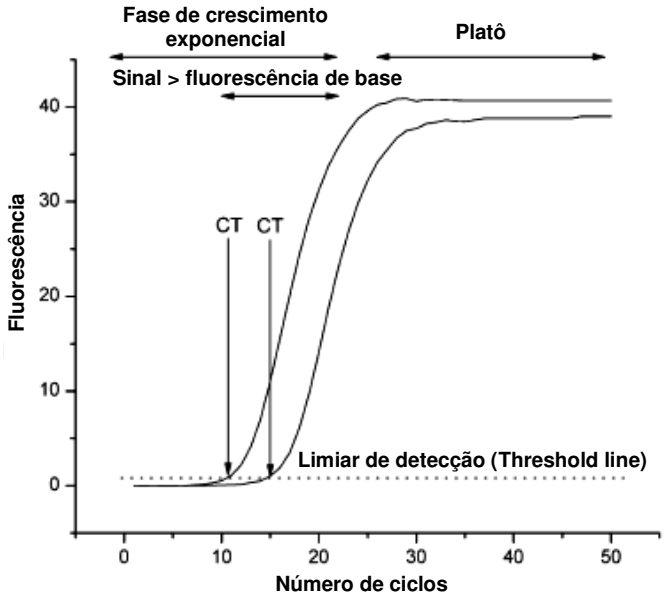

Figura 16. Curvas de amplificação gerada pela PCR, em Tempo Real. Modificado (KUBISTA *et al*., 2006).

Na PCR em Tempo Real os dois sistemas mais usados são TaqMan e SYBR Green. A primeira sonda fluorescente desenvolvida para essa reação foi a 5' nuclease, comumente referida pelo nome de TaqMan. Esta sonda é um curto oligonucleotídeo

(DNA) que contém um fluoróforo reporter 5' terminal semelhante à fluoresceína e um fluoróforo quencher 3' terminal vinculado (KAEDA *et al*., 2002; VALASEK *et al.,* 2005).

O SYBR Green é um corante que se liga à porção menor da fita dupla de DNA, tornando-o fluorescente. Este é o mais utilizado em reação de PCR em Tempo Real sendo capaz de identificar seqüências específicas de dupla-fita de DNA (dsDNA, *double-stranded*). A sua fluorescência não é detectada quando o mesmo não está vinculado ao dsDNA e apresenta uma afinidade pelo DNA, 100 vezes maior, em relação a do brometo de etídio, utilizado na PCR convencional. Durante os ciclos consecutivos da PCR, a quantidade de DNA de fita dupla se eleva de maneira exponencial, aumentando assim a quantidade de SYBR Green ligado e, subseqüentemente, sua fluorescência (WITTWER *et al*., 1997).

As vantagens da utilização do SYBR Green I incluem o fácil manuseio e o baixo custo. Enquanto que, a desvantagem para SYBR Green I é que o mesmo se liga a qualquer dsDNA, tais como produtos de amplificação não-específicos e dímeros de *primer*. Produtos não-específicos quando amplificados afetam a eficiência da amplificação de produtos específicos. Nesse contexto, a análise deve ser otimizada de forma a que a amplificação dos produtos não-específicos não ocorra (MORRISON *et al*., 1998).

Após a PCR, uma curva de *melting* é gerada por um aquecimento lento da dupla fita amplicom/sonda (heteroduplex) medindo a mudança na fluorescência que resulta quando a sonda desnatura, ou “melts”, fora do *amplicom*. A formação dessa curva é um exemplo de boas práticas para controlar a formação de dímeros iniciadores. A fluorescência é medida em função da temperatura, diminuindo gradualmente com o aumento da mesma (NYGREN *et al*., 1998). No entanto, ao atingir a temperatura a que

o dsDNA separa, o corante desloca e a fluorescência cai abruptamente (RIRIE *et al.*, 1997). Uma vez otimizada, a detecção SYBR Green I é altamente sensível para a identificação de um único alvo molecular na mistura da reação. O DNA-complexo resultante absorve a luz azul (λ max = 488 nm) e emite luz verde (λ max = 522 nm) (ZIPPER, 2004) (Figura 17).

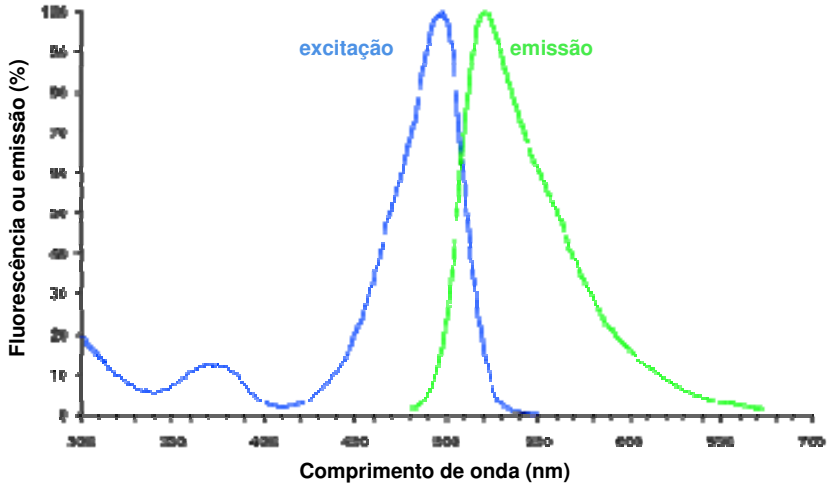

Figura 17. Espectrograma do SYBR Green I. Modificado (ZIPPER, 2004).

As principais vantagens da PCR em Tempo Real, em comparação com outros métodos de quantificação dos ácidos nucléicos são a extrema dinamicidade da reação (mais de oito ordens de grandeza) e uma significativa confiabilidade dos resultados obtidos, em comparação com a PCR convencional, pelo fato de que todo o perfil da amplificação é conhecido. Além disso, reações específicas, as quais a eficiência da amplificação é desviada (Ex.: presença de inibidores da polimerase) pode ser facilmente identificada pela reação da PCR em Tempo Real (WILHELM & PINGOUD, 2003).

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3. OBJETIVOS

3.1 Objetivo Geral

Determinar polimorfismo no gene da MBL-2, em mulheres infectadas por *C. trachomatis*, com e sem lesões precursoras do câncer de colo uterino, atendidas no Ambulatório Especializado da Mulher da Prefeitura Municipal do Recife, Pernambuco.

3.2 Objetivos Específicos

- Relatar uma revisão da literatura baseada na análise do perfil imunológico do hospedeiro, frente à infecção pelo HPV e *C. trachomatis*, cofator do câncer cervical;

- Detectar a presença da *C. trachomatis*, em secreções vaginais de mulheres brasileiras, pela técnica da PCR em Tempo Real, usando o SYBR Green I;

- Determinar a presença de polimorfismo no primeiro éxon do gene MBL-2, em mulheres brasileiras *C. trachomatis* positivas, a fim de correlacioná-lo com o aumento da susceptibilidade à infecção por esse cofator do câncer cervical, utilizando a técnica da PCR em Tempo Real.

4. ARTIGOS

4.1. Artigo nº 01

Immunological's profile host of HPV e *Chlamydia trachomatis*, cofactor of cervical cancer
Fator de Impacto 2.605
Artigo submetido à Human Immunology

4.2. Artigo nº 02

DETECTION OF *Chlamydia trachomatis* IN BRAZILIAN WOMEN BY REAL TIME POLYMERASE CHAIN REACTION USING SYBR Green I
Fator de Impacto 3.708
Artigo a ser submetido ao Journal of Clinical Microbiology

4.2. Artigo nº 03

Polymorphisms in MBL-2 gene in women infected by *Chlamydia trachomatis,* using Real-Time PCR
Fator de Impacto 2.605
Artigo a ser submetido à Human Immunology

4. ARTIGOS

4.1. Artigo nº 01

Immunological's host profile of HPV e *Chlamydia trachomatis*, cofactor of cervical cancer

Fator de Impacto 2.605

Artigo submetido à Human Immunology

Immunological's host profile of HPV and *Chlamydia trachomatis*, cofactor of cervical cancer

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Summary

Over 100 different genotypes of HPV, non-enveloped double-stranded DNA virus, have been isolated to date, and more than 40 of these types infect the epithelial and mucosal lining of the anogenital tract and other areas. By another side, *Chlamydia trachomatis* is one of the most common sexually transmitted agents that cause a wide spectrum of diseases including uterine cervical lesions, limphogranuloma, trachoma, conjunctivitis and pneumonia. Despite the presence of HPV and *C. trachomatis* in cervical samples, the exact relationship between these two organisms in genital infection remains not completely understood. It is possible that *C. trachomatis* infection became an independent factor or cofactor for HPV establishment and the development of CIN. Together with endogenous hormones, genetic factors, immunological

approaches and sexual behavior can also show synergism in inducing CIN.

Keywords: HPV*, Chlamydia trachomatis,* Cancer, Immunology.

Abreviations

APC

antigen-presenting cells

CIN

cervical intraepithelial neoplasia

bp

base-pair

C. muridarum

Chlamydia muridarum

C. pneumonia

Chlamydia pneumonia

C. trachomatis

Chlamydia trachomatis

DC

dendritic cells

DNA

acid deoxyribonucleic

EBs

elementary bodies

GM-CSF

granulocyte macrophage-colony stimulating factor

GSK

glaxosmitkline

HSP

heat shock protein

HBD-β

human β-defensins

HIV

human immunodeficiency virus

HPV

human papillomavirus

HSV-2

herpes simplex virus-2

IDO

indoleamine 2,3-dioxygenase

IFNs

interferons

IFN-α

interferon-α

IFN-β

interferon-β

IgG

immunoglobulin G

IL-1

interleukin-1

IL-1

interleukin-1

IL-2

interleukin-2

IL-6

interleukin-6

IL-12

interleukin-12

LC

Langerhans cells

LPS

lipopolysaccharide

LSIL

low-grade squamous intraepithelial lesions of the cervix

MBL

mannose-binding lectin

MH

ministery of health

MHC

major histocompatibility complex

MIF

microimmunofluorescence microscopy

MOMP

major outer membrane protein

NOD

nucleotide-binding oligomerization

OMP2

outer membrane protein-2

ORF

open reading frame

PAMPs

pathogen-associated molecular patters

PmpD

polymorphic membrane protein-D

pRb

retinoblastoma protein

PRRs

pattern recognition receptors

RBs

reticulate bodies

SILs

squamous intraepithelial lesions

SNP

single nucleotide polymorphisms

STIs

sexually transmitted infections

Tc

T –cytotoxic cell

Th1

T-helper 1 cell

Th2

T-helper 2 cell

TLRs

toll-like receptors

TNF-α

tumor necrosis factor-α

TNF-β

tumor necrosis factor-β

UK

United Kingdom

URR

upstream regulatory region

USA

United State of America

VLPs

virus like-particles

WHO

world health organization

Immunological Profile in Infections

The world is a dangerous place in with *Homo sapiens* must continuously utilize sophisticated, flexible, and lethal defenses to rebuff the massed regiments of viruses, bacteria and eukaryotic parasites [1]. It is traditional to organize host responses to infection into separate arms or compartments, such as complement, phagocytes, cytokines, cell-mediated immunity and humoral immunity. A more current approach has been considered in 2 larger categories: (i) innate immunity, incorporating the faster responses to infection and phylogenetically primitive and non-specific, such as surface defenses, cytokine elaboration, complement activation (classical, alternative and mannan-binding lectin (MBL) pathways) and phagocytic responses, and (ii) adaptative immunity, involving more slowly developing, long-lived, and highly evolved antigen-specific protective responses, such as antibody production and cell-mediated immunity, that exhibit extraordinarily diverse ranges of specificity [2].

The epithelium of the skin and mucous tissues acts as a barrier mechanism for the invasion of microbial pathogens. These epithelial cells are the largest source of antimicrobial peptides (β-defensins, lysozyme and cathelicidin) able to play an important defense in host, mainly against bacteria and virus. Studies in its structure, origin, expression and actions have also been revealed and correlated with their immunological activities, such as antimicrobial natural in their duties [3]. They are present in many mucous areas throughout the body and develop an important role as susceptible indicator to infection by the beginning of early activation of immune response in host. In a special way, the mucosal innate immune system of the female reproductive tract is uniquely adapted to facilitate specialized physiological functions that include menstruation, fertilization, implantation, pregnancy and parturition [4].

Innate immune system has critical importance in preventing microbial penetration. When a pathogen enters the body, epithelial cells are the first line of defense. Epithelial cells and circulating cells of the innate immune system possess cell-surface-bound or intracellular pattern recognition receptors (PRRs). The two most important families of PRRs are the toll-like receptor (TLR) family and the nucleotide-binding oligomerization domain (NOD) proteins. PRR recognize and bind pathogen-associated molecular patterns (PAMP), which are components on and in foreign organisms. Subsequently, macrophages, neutrophilis and dendritic cells (DCs) ingest the pathogen by phagocytosis and destroy it within the cell. Macrophages and DCs are able to express pathogen components (antigens) bound to major histocompatibility complex (MHC) proteins (also known as human leukocyte antigens) on their surface. These antigen-presenting cells (APCs) help the activation of the acquired immune system [2].

The immunity against bacterial infections is extremely complex because of several virulence factors used by bacteria to enhance its survival. Indeed, many, if not most of bacteria virulence factors, is intended to prevent or neutralize the host's defenses. The primary nonspecific defense against bacterial infections is provided by granulocytes, which ingest and kill the majority of potential pathogens [5]. The specific immunity is necessary to protect the host front of intracellular capsulated bacteria by promoting the development of antibodies, which can increase the process of destruction through opsonization activity, fixing of completion or the immunity of T-cells, capable of activating macrophages [6]. During infection, the bacteria can be phagocytosed by APCs. In such cells, the bacteria-containing phagosome are fused with lysosomes, the bacterial antigens are processed and eventually peptide fragments are presented in the groove of human histocompatibility antigen class II (MHC II) at the cellular surface, where they will stimulate a T-helper cell response. Such bacterial-specific CD4+ T-cells are activated after recognition of the peptide-MHC-class II complex. The activated CD4+ T-cells play a role as

inhibitors of bacterial replication and as stimulators of protective immunity involving immune and inflammatory cells [7]. Nevertheless, the major function of CD4+ helper T-cells is to promote the activation of B cells, CD8+ T-cells, and other inflammatory cells, both by contact-dependent and cytokine-mediated processes [8].

The basic model of the immune response against virus involves, primarily, processing and presentation of viral antigens by Langerhans cells (LCs) or other APCs. On the surface of these APCs is found a particular group of PRRs that recognize PAMP in the structure of the virus. TLRs are also critical in the regulation of the production of type 1 IFNs, which are important components of the host defense against viruses. Finally, by virtue of their intracellular life cycle, viruses have a much greater opportunity than bacteria to interact with many different TLRs, and they have developed a number of methods of inhibiting TLR-specific responses [9]. Along with this recognition, between PRR and PAMP, the connection of pro-inflammatory cytokines to LCs seem to be of fundamental importance, because activate the processing of antigen and also their migration to the lymphnodes. During the migration, the LCs become mature and highly specialized in the presentation of antigens by molecules of the major histocompatibility complex class I (MHC I) and able to activate the virgin T cells, adaptive immune response, in these secondary lymphoid organs [10].

The adaptive immune response generates specific lethal effectors responses to foreign antigens as well as long-lived cells with memory of the insult. Antibody-mediated humoral immunity clears free virus particles from body fluids and normally can prevent viral reinfection, while cell-mediated immune responses are essential for the clearance of virus-infected cells and the generation of immune memory [11].

The adaptative immune system consists of a humoral arm (with B lymphocytes, mainly targeting extracellular pathogens) and a cell-mediated arm (with T lymphocytes, mainly targeting

intracellular pathogens), which closely interact. In the humoral arm, B lymphocytes are activated by APCs (cells of the innate immune system or T lymphocytes), which develop itself into plasma cells and produce antibodies that neutralize the antigen or directly destroy the pathogen. In the cell-mediated arm, T lymphocytes are activated by APCs (cells of the innate immune system or B lymphocytes). Most T lymphocytes are T helper (Th) cells that produce pro-inflamatory cytokines. The Th1 subclass produces interleukin (IL)-12 and interferon-γ which support the cell-mediated system. The Th2 subclass produces IL-4, IL-5, IL-6 and IL-10, which support the humoral system [12].

Cytotoxic T (Tc) cells (or killer cells) directly attack and destroy a pathogen and produce pro-inflammatory cytokines. The secretion of chemokines and pro-inflammatory cytokines, such as IL-1, IL-1α, IL-6, tumor necrosis factor-α (TNF-α) and granulocyte macrophage-colony stimulating factor (GM-CSF) produced by epithelial cells start the recruitment of the cells from immune system to the epithelial/mucosal surface, allowing the activation of macrophages and DCs and the differentiation of lymphocytes effectors [13]. Besides these, the IL-18 together with the IL-12 regulate the responses of the Th1 cell cytokines, expressed in cells lining the gut, important in the differentiation of cells [14].

Human Papillomavirus overcomes the immunological system

Papillomaviruses are ubiquitous and have been detected in a wide variety of animals as well as in humans, being specific for their respective hosts. During the past 25 years, the understanding on papillomaviruses in general and human papillomavirus (HPV) in particular has increased dramatically. This virus is linked to the precursor lesions in the uterine cervix and the

progression to invasive cervical carcinoma. This is the second most common malignancy in women worldwide, with an estimated incidence of 500 000 cases per year and 250 000 deaths [15].

HPV particles consist about 8000bp long circular deoxyribonucleic acid (DNA) molecules wrapped into a protein shell that is composed by two molecules (L1 and L2). The genome has coding capacity for these two proteins and at least six so-called early proteins (E1, E2, E4-E7), which are necessary for the replication of the viral DNA and for the assembly of newly produced virus particles within the infected cells. Both sets of genes are separated by an upstream regulatory region (URR) of about 1000 bp that does not code for proteins but contains *cis*-elements required for regulation of gene expression, replication of the genome, and its packaging into virus particles [16].

Over 100 HPV types were identified so far, about 40 of these have being considered genital HPV types and classified into low-risk, intermediate-risk and high-risk classes, based on their association with cervical cancer and precursor lesions [17,18,19,20]. Low risk types include HPV-6, 11, 42, 43, and 44, while high risk types include HPV-16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, and 70. However, these HPV genotypes have come under renewed classification, with HPV-68 characterized as “probable high-risk”, together with HPV 26, 53, 66, 73 and 82 [21]. On the other side, HPV-6 and 11 are associated with the majority of more benign lesions affecting the anogenital areas, such as genital warts (condylomata) and low-grade squamous intraepithelial lesions of the cervix (LSIL) and vulva [22].

The fact that HPV is a prerequisite for invasive cervical cancer implies that other risk factors may be considered to be cofactors rather than independent factors. These cofactors would act by influencing HPV acquisition, its persistence, and the development of a neoplastic lesion [23]. Three groups of potential cofactors are: (1) environmental or exogenous cofactors,

including dietary deficiencies, hormonal contraceptives, tobacco smoking, parity, and coinfection with other sexually transmitted agents *(C. trachomatis* and herpes simplex virus type-2 – HSV-2, immunosuppressant); (2) viral cofactors, such as infection by specific types, coinfection with other HPV types, HPV variants, viral load, and viral integration; (3) host cofactors, including endogenous hormones, genetic factors, and other factors related to the immune response [24].

Much has been learned concerning mechanisms by which HPV causes cancer, overcoming the immune system. HPV has tissue tropism and is able to infect primitive basal keratinocytes, probably targeting stem cells, but only expresses high levels of viral structural proteins and viral assembly in the upper layers of the stratum spinosum and granulosum of the squamous epithelia. Oncogenic HPV strains, which can cause persistent infection, can remain in episomal way or integrate randomly into the host genome. In this case, viral genome disrupts inside E2 open reading frame (ORF), leading to a loss of transcriptional control of both E6 and E7, leading to the persistent expression of these oncoproteins. Over-expression of these two HPV non-structural genes appears to be associated with enhanced E7 interactions with the retinoblastoma protein (pRb) that promote cell proliferation and E6 enhancement of the degradation of p53, which inhibits apoptosis allowing the development of carcinoma [25,26,27].

Regression of viral-infected cells requires cell mediated immunity. MHC expression can be altered and antigen recognition disturbed by genes products encoded by DNA viruses, providing the virus with a selective advantage. HPV-infected cells show suppression of class I MHC expression that may be virally direct. HPV may contribute to the loss of responsiveness of infected cells to potent MHC-I inducing cytokines and MHC gene expression can be influenced, either directly or indirectly, by the presence of HPV. Expression of E7 or E5 early genes, which seem to interact with the antigen-processing system *in vitro*, might exert a direct influence [28].

The infectious cycle of HPV is itself an immune evasion mechanism inhibiting host detection of virus. Clinical evidences indicate that viral gene expression is confined to the keratinocyte or cells with the potential for squamous maturation. There is no cytolysis or cytopathic death as a consequence of HPV replication, assembly, or viral particle release because the keratinocyte is a cell destined for death and desquamation, far from the sites of immune surveillance [29,30]. In this way, HPV replication and release do not cause cell death. Considering that cell death is a danger signal used as a prerequisite for inflammation, HPV infectious cycle causes little or no release of the proinflammatory cytokines important for DCs activation and migration into the local. These activations are essential signals required for immune responses in squamous epithelia are absent, and avoiding this response virus remains in the host organism without being eradicated by the immune system [31].

Immunological studies showed that the number of LC was significantly reduced in dysplasia or HPV infection, consistent with an early failure of endogenous activation events influenced by viral gene expression [32]. Others studies have also show a lack of activation of LCs in cervical lesions, together with a down regulation of the cytokine TNF-α produced by basal keratinocytes, and an up-regulation of the suppressive cytokine IL-10 in cervical intraepithelial neoplasia (CIN) compared to normal cervical epithelium [33]. In addition, high-risk HPV E6 and E7 oncoprotein modulate the IFN-α response pathways of the infected cells and thereby compromise any protection from interferon's induced by innate immunity [34,35]. Evidence for increased papilloma incidence in T-cell immunosuppressed patients strongly suggests that CD4+ and/or CD8+ T-cell responses play a vital role in controlling infection with these agents [36].

It is possible that changes in the host response to HPV, that occur during concurrent infection, may decrease the host's ability to resolve the infection. Persistence of HPV infection has been closely associated with progression to cancer. In addition, infection with *C. trachomatis* facilitates the transmission of HIV and might be a co-factor in HPV-induced cervical neoplasia [37, 38].

Chamydia thachomatis as potential cofactor for cervical lesions

C. trachomatis is a Gram-negative microorganisms, non-motile, coccoid, aerobic, obligate intracellular pathogen, with a distinctive dimorphic life cycle. Because they are unable to synthesize their ATP, they have to use their host cell's energy resources. For this reason *C. trachomatis* were once considered viruses [39]. The life cycle of *C. trachomatis* occurs in two distinct periods. So, the *C. trachomatis* elementary bodies (EBs) infect non-ciliated columnar cells and macrophages. Once in contact with the surface of the host cell, *Chlamydia* induces its own endocytosis. Inside the cell, the EB germinate and form the reticulate bodies (RBs) that, after an incubation of 7-21 days, begin to replicate every 3 hours. The RB produced converts into EB and shed off the cell membrane through exocytose. It is thought that *Chlamydia's* ability to survive phagocytosis and destruction by lysosomal enzymes is due to its unique cell-wall structure. This cell-wall is rich in cysteine and lipopolysacharide, and protects *Chlamydia* inside and outside the cells [40].

This bacteria is one of the most common sexually transmitted agents which cause a wide spectrum of diseases including uterine cervical lesions, venereal limphogranuloma, trachoma, conjunctivitis and newborn pneumonia in humans [*41*]. These kinds of diseases can be caused by several serovariants of *C. trachomatis* based on the features of their major membrane proteins.

The serovars A, B and C cause trachoma; serovars D-K infect ophthalmic, genital and rectal columnar epithelial cells leading to conjunctivitis, urethritis, cervicitis and prostatic, respectively [42]. Prevalence of *C. trachomatis* infection in lower genital tract is 2-25%, being the highest in young people. The World Health Organization (WHO) has estimated 89 million new cases of genital *Chlamydial* infections worldwide in 1995 and 92 million in 1999 [43,44].

Regarded to intracellular infection by *C. trachomatis*, several steps are required in order to successfully establish infection, to replicate and to spread. Each stage presents an opportunity for one or more elements of the immune system to block unrestricted growth of the organism [45]. Intracellular PRR have been identified as involved in *C. thracomatis* infections, as TLR and NOD proteins. So far, 11 different TLRs have been identified not only intracellular but in cell-surface-bound. TLR2 is specific for *C. trachomatis* component peptidoglycan, and TLR4 is for *C. trachomatis* components lipopolysaccharide (LPS) and heat shock protein (HSP). TLR1, TLR3, TLR5 and TLR6 are also present in the human female genital tract, but they do not recognize *C. trachomatis* PAMP [46]. This suggests that these TLRs may play a role in the host defense against non-*C. trachomatis* and/or polymicrobial genital tract infections. NOD proteins are intracellular PRR. Because *C. trachomatis* is an intracellular pathogen containing LPS and peptidoglycan, a role of intracellular NODs in the recognition of *C. trachomatis* has been suggested [47, 48, 49].

Immunity after a natural infection is not completely effective, as previous exposure to *C. trachomatis* offers only limited protection against re-infection. Current research on acquired immunity to *C. trachomatis* has focused largely on the central role of T lymphocytes in orchestrating the multiple immune mechanisms required to achieve protective immunity. There is strong evidence for the involvement of cell-mediated immunity (Th-1 pathway) in resolving a *C. trachomatis* infection together with its associated cytokines, IL-2, IL-12 and IFN- [50,51,52]. It

has consistently been demonstrated that IFN- has been shown as able to inhibit the growth of *C. trachomatis* in cell culture. This molecule cans upregulate macrophage phagocytes potential and the expression of MHC molecules by both professional and non-professional APCs, leading to enhanced presentation of microbial antigens to both CD4+ and CD8+ T-cells, being critical for protection [53,54,55].

The protection against *C. trachomatis* is serovar-specific and can be attributed largely to antibody specific for the major outer-membrane protein (MOMP), the primary determinant that defines a serovar [56]. Antibodies to *C. trachomatis* are directed against surface components such as the genus-specific LPS and MOMP [57]. Other major antigens are the cysteine-rich outer membrane protein 2 (OMP2) and the *Chlamydial* homologues to HSP60 and HSP70 (*Chlamydial* Hsp60/GroEL and *Chlamydial* Hsp70/DnaK) [58]. OMP2 is localized in the outer membrane of *Chlamydiae* but is debated whether it gets access to the surface [59,60].

There is significant data demonstrating that both CD4+ and CD8+ T-cells are involved in controlling *C. trachomatis* infection. In human and animal models, both T-cell subsets are detected at the site of *C. trachomatis* infection [61]. Several *C. trachomatis* antigenic peptides capable of stimulating CD4+ T-cells have been identified. CD4+ T-cell lines from infected patients have thus been shown to be stimulated by MOMP, OMP2, GroEL and PmpD, a protein belonging to the polymorphic membrane protein family. Enolase and YopD ortolog have also been shown to active CD4+ T-cells [62,63].

Relevant strategies of avoidance of *C. trachomatis* are based on the biosynthesis of tryptophan to escape the mechanism of defense of interferon and allelic variation of their dominant surface protein – MOMP. Because of MOMP is considered as an important target of immunity, the immunity specificity of different serotypes of *C. trachomatis* could be studied in the model *C. muridarum* in mice. Moreover, given that the *C. trachomatis* is a major cause of

immune-mediated damage in the tract reproductive of patients infected, *Chlamydial* antigens and the host genetic factors have been identified as contributors in immunologic and pathologic events. However, a better understanding of the specific components involved the destruction *vs.* protective immune response to infections by *C. trachomatis* is still far from being well elucidated [64].

Since infection with HPV or *C. trachomatis* were linked with the development of cervical cancer, a number of studies investigated the relationship between past infection with HPV or *C. trachomatis* on the subsequent infection with the other agents [65,66,67,68]. This fact was exemplified by the demonstrations that *C. trachomatis* was found in samples from the cervix of HPV-positive women. *C. trachomatis* DNA or IgG antibodies, were also higher in HPV-positive compared to HPV-negative women. It is possible that *C. trachomatis* infection may be an independent factor or cofactor for HPV in the development of invasive cervical carcinoma. Nevertheless, the exact relationship between *C. trachomatis* and HPV infection remains not completely understood [69,70].

The identification of novel immune-evasion strategies and the analysis of their functions in the context of a bacterial and viral infections should lead to a better understanding of the immune system and the interaction of them with their hosts. Besides studying natural immunity, another equally important approach is to develop immunization strategies that prime the arms of the immune response that are most effective at reducing the replicative capacity of these organisms, whether or not these responses are dominant during natural infection [71].

Immunological approach against HPV and *Chlamydia trachomatis*

Vaccine development is needed for both microorganisms, but implementation as a rational public health intervention will be complex and depend upon social and economic factors. Prophylactic vaccines have been the most effective strategy for controlling viral infections and the evidence is accumulating; and HPVs are no exception to this fact [14].

HPV vaccines developed up to now are based in subunit vaccines consisting only L1 protein assembled into macromolecular structures known as virus-like particles (VLPs). Early studies in animals indicated that immunization with purified virions could induce protection and these observations were followed by studies in a number of laboratories indicating that recombinant viruses like-particles (VLPs). These particles have the structure and antigenic characteristics of virions and could be produced by expression of L1 or L1/L2 in high yield eukaryotic expression systems, such as yeast or *Baculovirus* [72]. The ability to produce HPV-VLPs in large amounts has resulted in promising phase II clinical trials focused to prevent infection of sexually active young women with highly oncogenic strains of HPV. In these studies, VLPs derived from selected oncogenic strains of HPV were administered by intramuscular injection. A yeast expressed vaccine developed by Merck® contains VLPs derived from the two most common oncogenic HPV types, 16 and 18, as well as from two common genital wart types, 6 and 11 [73]. Similar Phase II results have been observed using a candidate vaccine developed by GlaxoSmithKline® (GSK) and their partners containing HPV-16 and 18 VLPs produced by insect *Spodoptera frugiperda* SF9 cells and adjuvanted with AS04, a mixture of aluminum hydroxide and 3 deacetylated monophosphoryl Lipid A. Very high levels of protection against

type HPV-16 and 18 infection as well as HPV-associated cervical histological abnormalities (93%) were observed in young women up to 27 months following vaccination [74].

Both vaccines have undergone randomized placebo-controlled double-blind clinical trials in women in North America, Latin America, Europe and Asia Pacific and have been granted a license in many countries and for the European Union by the European Medicines Evaluation Agency. The quadrivalent vaccine has been licensed by the Federal Drugs Agency of the USA since June 2006 [75].

The observation that the immune response is directly or indirectly involved in the pathogenesis of disease caused by *Chlamydia spp.* also introduces further complexity to the vaccine-development process. Nonetheless, the substantial progress that has been made in elucidating the immunobiology of *C. muridarum* infection is greatly facilitating a renewed effort to design a vaccine against infection with *C. trachomatis*. Selection of defined antigens for a recombinant subunit vaccine that stimulates CD4+ Th1-cells is central to the current design strategy. Nevertheless, progress has been achieved in the past few years and has led to the identification of various protective *C. trachomatis* antigens, OMP2, HSP60, YopD homologue (homologue of *Yersinia pseudotuberculosis,* YopD), enolase and PmpD (polymorphic membrane protein D), as potential vaccine candidates [76]. The OMP2 is also an immunodominant antigen that contains CD4+ and CD8+ T-cell epitopes. It is more highly conserved in amino-acid sequence among different *C. trachomatis* serovars than MOMP; therefore, in a vaccine, it could provide protection against the different *C. trachomatis* serovars. Recent experiments have shown that inclusion of OMP2 considerably improves the protective potential of MOMP-based vaccines. However, whether these T-cell antigens provide immune protection remains to be determined [77,78].

Conclusion

In higher organisms a variety of most defense mechanisms control the resident microflora and, in most cases, effectively prevent invasive microbial disease. However, it appears that microbial organisms have coevolved with their hosts to overcome protective host barriers and, in selected cases, actually take advantage of innate host responses. Many microbial pathogens avoid host recognition or dampen the subsequent immune activation through sophisticated interactions with host responses, but some pathogens benefit from the stimulation of inflammatory reactions.

It is probable that HPV and *C. trachomatis* are covariables/cofactors related to sexual behavior as shown here and elsewhere, that they synergized in inducing CIN, as was suggested earlier. Although the study addressed *C. trachomatis* infection in established HPV cases, and hence could not determine the exact cause-and-effect relationship between *C. trachomatis* and HPV infection which influences the outcome of the course of the infection, it points to a possible association between HPV and *C. trachomatis* infections with the incidence of invasive cervical cancer by inducing expression of proinflammatory mediators, altering cell-to-cell adhesion, and affecting cellular differentiation. Nevertheless, the association between HPV and *C. trachomatis* infection remains to be understood.

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4. ARTIGOS

4.2. Artigo nº 02

DETECTION OF *Chlamydia trachomatis* IN BRAZILIAN WOMEN BY REAL TIME POLYMERASE CHAIN REACTION USING SYBR Green I

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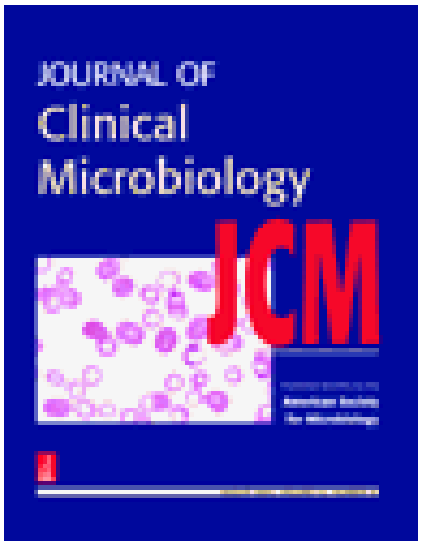

DETECTION OF *Chlamydia trachomatis* IN BRAZILIAN WOMEN BY REAL TIME POLYMERASE CHAIN REACTION USING SYBR Green I.

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The aim of this work was purpose a methodology for the identification and diagnostic of *Chlamydia trachomatis (C. trachomatis)* based in the technology of Real-Time PCR assay using SYBR Green I as a flourescence dye. A total of 98 clinical samples (vaginal secretion) were obtained during routine examination in the Specialized Ambulatory of Women, Recife, Pernambuco State. In the population study, 14,3% (n=14) of the samples were positive for *C. trachomatis* using this thecnique. Those samples was confirmed by melting temperature assay. There was more than one peak of melting, but the specific melting temperature was 77±0,5 and it was confirmed with analyses of 1% agarose gel electrophoresis that showed one band of approximately 150 bp DNA indicating the presence of the bacteria and validation the PCR results. The other side, the inespecific peak of melting was associated with dimer primers. This study found a prevalence of 14,3% women infected with *C. trachomatis.* The results showed that the present Real-Time PCR assay was of high specificity and sensitivity. This assay may therefore offer a rapid and reliable means for screening of the *Chlamydiaceae* pathogens.

Chlamydia trachomatis is a bacteria that causes infection of global public health significance that is considered to be one of the major causes of Sexually Transmitted Infections (STIs) worldwide [8, 24]. The bacteria is a Gram-negative, pleomorphic, non-motile organism about 0.2-1.5µm in length. Because they are unable to synthesize their ATP, they have to use host cell's energy resources [17]. The number of these infections is displaying an increasing tendency worldwide [5]. The prevalence of *C. trachomatis* genital infections in women has been reported ranging from 0 to 37%, depending on the population studied and the techniques used. Several risk factors play a role in this tendency: age under 25, family status, education, beginning of sexual activity and number of sexual partner among others [1, 14].

According to estimates from the World Health Organization (WHO), it occurs each year 4 million new cases of infection in the United States, 10 million in Europe and 92 million around the world [25]. In Brazil, there are few data on its prevalence, but according to the Ministry of Health (MS) in 2001 occurred about two million cases [18]. An estimated 3 million *Chlamydia*

trachomatis infections occur annually among sexually active adolescents and young adults in the United States [9]. The majority of persons with *C. trachomatis* infection are not aware of their infection because they do not have symptoms that would prompt them to seek medical care. Consequently, screening is necessary to identify and treat this infection [23].

In women, urogenital *C. trachomatis* infections can cause a broad spectrum of clinical manifestations; including urethritis, cervicitis, and pelvic inflammatory disease, which if untreated may lead to serious complications, including ectopic pregnancy and tubal infertility. Many women do not seek medical care, since more than 70% of genital infections are asymptomatic [3, 11].

A variety of diagnostic tests are currently available for the diagnosis of *Chlamydia trachomatis* infections: like cell culture, antigen detection methods, including enzyme immunoassay (EIA) and direct immunofluorescence (DFA), DNA probes, and Nucleic Acid Amplification Tests (NAATs) [15]. Molecular diagnostic techniques, such as PCR, are being developed to aid in the diagnosis of *C. trachomatis* infection by detecting bacterial genetic material. Unlike culture, most molecular assays are designed specifically for one organism. This provides high sensitivity and specificity but detects only what you are looking for; multiple assays may be required to screen for multiple organisms. Broad-range assays, based on ribosomal genes (rDNA), are designed to overcome this limitation [10]. There are many highly conserved nucleotide sequences in different chromosomal genes of *C. trachomatis*. These sequences are used as targets for Nucleic Acid Amplification Tests (NAATs). NAATs have surpassed cell culture and antigen detection for the diagnosis of *C. trachomatis* infections due to their enhanced sensitivities [12]. An oligonucleotide array technology for rapid detecting and genotyping of *Chlamydia trachomatis* from urogenital specimens was established [27].

Real-Time PCR assay require a fluorescent-based method of signal generation to enable analysts to be detected in closed tubes. Many different strategies are available to the results researcher. Several previously reported real-time PCR assays for the detection of *C. Trachomatis*, most of methods are based on a fluorescent dye-labeled TaqMan® probe-based system and this system has many advantages [12, 16, 20].

We describe the development of a broad-range Real-Time PCR methodology for detection system for routine qualitative diagnosis of *C. Trachomatis* infection using the SYBR®GREEN Master Mix Real-Time PCR (*Applied Biosystems, Foster City, CA, USA*) as a fluorescence dye. This method assay was found to be specific, sensitive, fast and inexpensive for use in the routine diagnostic clinical microbiology service. In the present report, we describe a melting temperature assay protocol for cost-effective *C. Trachomatis*.

MATERIAL AND METHODS

Clinical samples. Sampling was carried out after obtaining the informed consent during epidemiological surveys, and in accordance with the Helsinki Declaration and the EEC human experimentation guidelines. A total of 98 clinical samples (vaginal secretion) were obtained during routine examination in the Specialized Ambulatory of Women, Recife, Pernambuco State. The significance of the study was explained to the patients, and informed consent was obtained. The study was approved by the Ethical Committee of the Instituto Materno Infantil de Pernambuco and by the Ethical Committee of the Associação Caruaruense de Ensino Superior, Faculty of Agreste of Pernambuco.

Collection of endocervical *swabs* and DNA extraction. Two cotton swabs of the cervical were obtained. The swabs were placed into a sterile 15mL collection tube containing 2mL of phosphate buffered saline and transported to the laboratory for further processing. Upon arrival, the swabs were vortexed vigorously. The swab fluid was centrifuged at 2.500 rpm for 10 min, the supernatant was removed, and the cell pellet resuspended. The DNA extraction was performed using Wizard®Genomic DNA Purification Kit (*Promega Corporation*), according with manufacturer's instructions. Following extraction of samples, each sample was analised to an 1% agarose gel stained with ethidium bromide.

Primers. The C*hlamydial* genes coding were performed. The forward primer (5´-GGGAGCAACTACCGGTTA-3´) and the reverse primer (5'-GTGTCTGTATAAAGCTCAACC-3'). The nucleotide BLAST program at www.ncbi.nlm.nih.gov/BLAST/BLAST.cgi demonstrated the unique sequence of this molecule.

Polymerase chain reaction (PCR). Real-time PCR was performed with a Rotor-Gene™ RG 3000 (*Uniscience-Cobert Research, Australia*). Rotor-Gene has four light-emitting diodes that allow the detection of up to four targets in a single amplification reaction. A pair of primers was used for each component of the assay (forward and reverse). PCR was performed in a 15µL reaction mixture containing 2µL of DNA (50ng) from a clinical sample (vaginal secretion), 7.5µL of 2 x SYBR®GREEN Master Mix Real-Time PCR (*Applied Biosystems, Foster City, CA, USA*), 1,5µM of each primer (10pmol/uL) and Milliq water *q.s.p.* 15µL. The amplification reaction profile included heating at 95°C for 4 min, followed by 40 cycles of 95°C for 1 s and 58°C for 60 s. The acquisition of a signal was performed at 58°C during each extension phase. After 40 cycles a melting curve was generated by heating the sample to 95°C programed followed by cooling down to 60°C for 15s and slowly heating the samples at 0,3°C/s to 95°C while de fluorescence is observed at the denaturing/melting temperature of a DNA fragment, which is a unique feature of that fragment. The specific PCR products having approximately 150 pb was confirmed on a 1% agarose gel stained with ethidium bromide.

Criteria for a positive result by Real-Time PCR. A sample was considered positive for *C. trachomatis* DNA if it produced signals in the channel. The sample was designated positive if repeat testing confirmed the earlier finding. Otherwise, it was designated a false-positive result. Moreover, 100% samples that were positive in the fluorescence direct were confirmed by Real Time PCR.

RESULTS

Analysis of curve to amplification of DNA *C. trachomatis*. The curve thershold (Ct) values are plotted versus the log of the initial amount of genomic DNA (Figure 1). No amplicon was generated in the sample without template showing specificity to reaction (basal line). In the population study, 14,3% (n=14/98) of the samples were positive for *C. trachomatis* by Real-Time PCR diagnostic. In addition, 85,7% (n=84/98) of the samples were negative for *C. trachomatis* by the same technique.

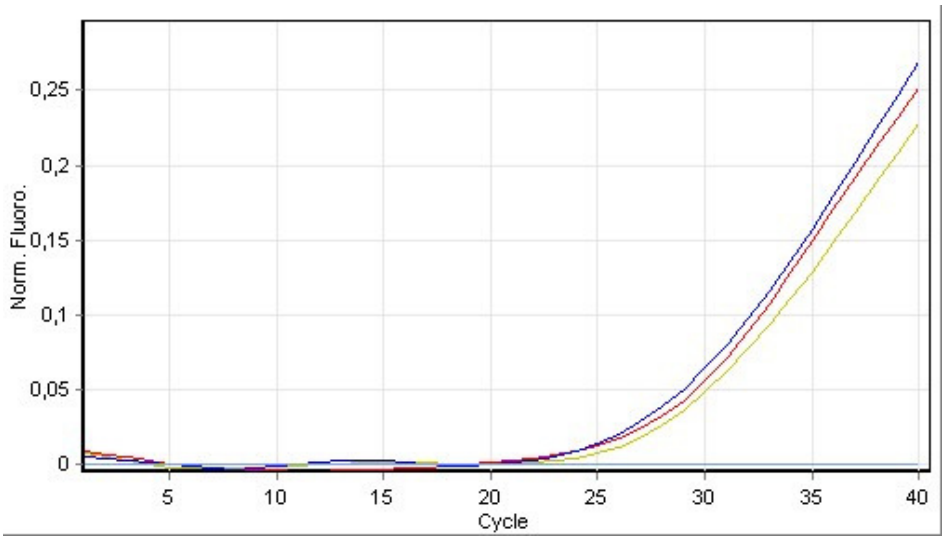

Figure 1. Amplification curve of *Chlamydia trachomatis* were acquired for PCR products formed after 15 cycles of amplification. No amplicon was generated in the sample without template showing specificity to reaction (basal line).

Melting-peaks analysis. To differentiate desired form undesired amplification products, melting peaks were acquired for each sample (Figure 2). Fluorescence data are acquired as the sample is heated at 0.2 °C/s from 58 °C to 95 °C. A plot of the ratio of 2 x SYBR®GREEN Master Mix Real-Time PCR (*Applied Biosystem, Foster City, CA, USA*) to fluorescence *vs*. temperature illustrates the melting properties. By plotting the negative derivative of the ratio with temperature *vs*. temperature, the data show peaks for where the maximum melting occurs. The figure 2 shows melting peaks obtained after amplification DNA of 77± 0,5, whereas the negative control didn´t shows none peaks.

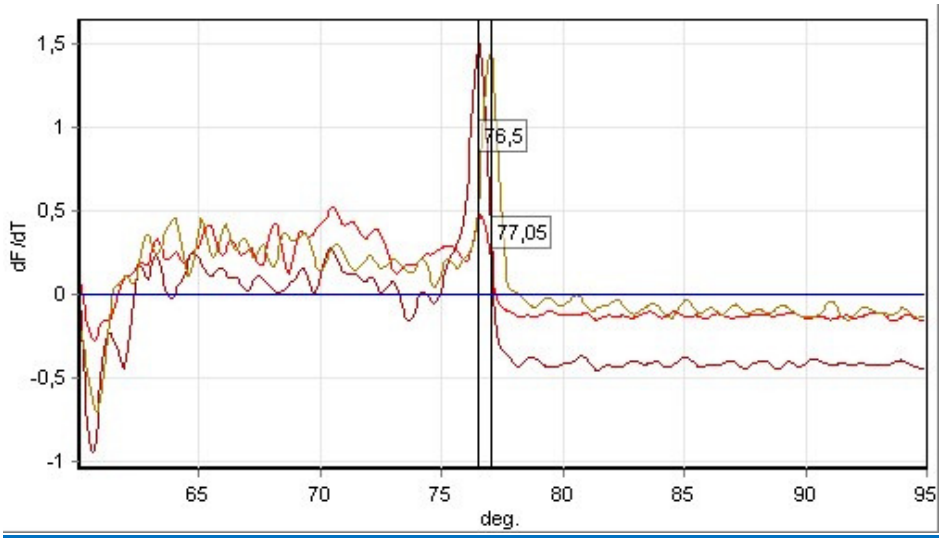

Figure 2. Meltting curve created by amplicons.

Analyses in agaroses gel at 1%. Undesired amplification products generated by melting peaks were confirmed using 1% agarose gel electrophoresis that showed that in addiction of a approximately 150 bp DNA band, there were primers dimers in PCR results (Figure 3).

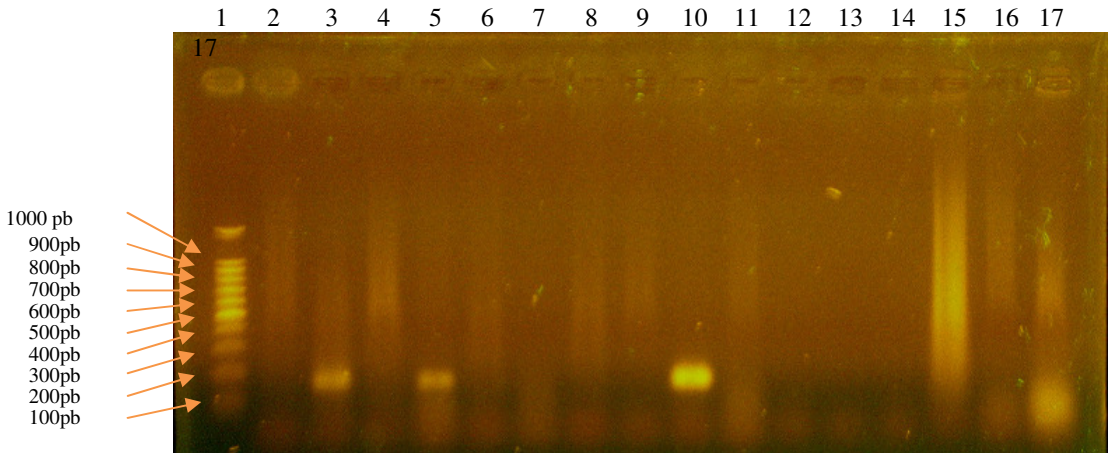

Figure 3. Analyses of amplification of *Clamydia trachomatis* by electrophoresis in 1% agarose gel. Line 1- Ladder 1000 bp; Lines 2 – 17 products of PCR by differents samples of vaginal secretion. The samples 3, 5 and 10 showed a specific fragment of 150 bp DNA in 1% agarose gel electrophoresis, indicating the presence of the bacteria and validation the PCR results.

DISCUSSION

PCR technology has been successfully employed during the last ten years for diagnosis of several microorganisms and has been proposed by different authors as an alternative methodology for C. *trachomatis* diagnosis [14, 15]. Many studies related to *C. trachomatis* prevalence using PCR from cervical scrapers or urine have been done [5, 7, 19], obtaining similar results. Other authors have compared for accuracy several methods like tissue culture or antigen detection with PCR and all of them have obtained excellent results with the latter, which has proven to have more sensitivity than tissue culture [4].

This study developed a methodology that was able to detect *C. trachomatis* using a Real-Time PCR assay. Many Real-Time PCR assays have been reported, however, most methods are based on a fluorescent dye-labeled TaqMan probe-based system and this system has many advantages [7,8,9]. However is worth to mention that in this work we used Real-Time PCR assays employed

SYBR Green I as fluorescence. It is a dsDNA-binding dye capable of binding to any dsDNA, including specific and non-specific PCR products and primer dimmers [13, 21]. Those could be differeciated by melting temperature assay technique. We showed that, with little optimization, SYBR®GREEN Master Mix Real-Time PCR (*Applied Biosystems, Foster City, CA, USA*) could be used as cheap and effective alternative.

In this study, 14,3% (n=14/98) women were infected with *C. trachomatis.* These findings are in agreement with most previous reports [2]. Shrier *et al.*, (2004) studied 126 patients by several methods of culture, PCR and LCR, using uretral secretion (PCR and culture), vaginal secretion (PCR), endocervical (Culture, PCR and LCR) and urine (PCR and LCR). The results showed that the present Real-Time PCR assay was of high specificity and sensitivity. This assay may therefore offer a rapid, economic and reliable means for screening of the *Chlamydiaceae* pathogens. The high prevalence of *C. trachomatis* and its severe impact on public health suggests the necessity of implement better diagnostic methods for its detection in the routine of STIs Laboratory Public Services in Pernambuco. PCR could be an adequate alternative method for diagnosis.

In conclusion, this Real-Time PCR method using SYBER Green I as flourescence provided a novel qualitative method for detection of *C. trachomatis*. Our work would provide an excellent choice for clinical diagnosis and monitoring of *C. trachomatis* infection.

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4. ARTIGOS

4.3. Artigo nº 03

Polymorphisms in MBL-2 gene in women infected by *Chlamydia trachomatis,* using Real-Time PCR

Fator de Impacto 2.605

Artigo a ser submetido à Human Immunology

Polymorphisms in MBL-2 gene in women infected by *Chlamydia trachomatis,* using Real-Time PCR

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SUMMARY

The present study looked for relationship between functional polymorphisms in the MBL-2 gene and host susceptibility to *C. trachomatis* infection in a Brazilian woman population. A total of 132 samples of DNA were extracted from vaginal secretions. Thirty and three samples were infected women and 99 healthy control subjects. Genotyping of MBL-2 human gene was performed using melting temperature assay, from the technology of Real-Time PCR. All samples were within the Hardy-Weinberg equilibrium (χ^2=2.65). Although, the allele 0 has been more frequent in the infected group than in the control group (36% *vs.* 26%, respectively) this difference wasn't significant (p=0.3112). Regarding to the genotypic frequencies, the genotype 0/0 didn't showed difference between infected group and control group (6% *vs.* 3%,

respectively) (p = 0.1430). However, the genotype A/0 presented a higher frequency in the group infected 61% in contrast to the control group 40% . Our data suggest that individuals carrying the functional polymorphisms in the first exon of MBL-2 gene do not have correlation with an increased chance of being infected with *C. trachomatis*.

Keywords: MBL-2; *Chlamydia trachomatis,* Genetic polymorphisms; Innate immunity.

ABREVIATIONS

ASCUS
: atypical squamous cells of undetermined significance

C. trachomatis
: *Chlamydia trachomatis*

HIV
: human immunodeficiency virus

MBL
: mannose-binding lectin

PCR
: polymerase chain reaction

RT-PCR
: Real-Time polymerase chain reaction

SNP
: single nucleotide polymorphism

STD
: sexual transmitted disease

WHO
: world health organization

INTRODUCTION

Real-Time Polymerase Chain Reaction (RT-PCR) is a method that allows to quantifying the amplification product. During Real-Time PCR, the accumulation of amplicons is detected in

Real-Time for each cycle of the reaction, through the excitement of fluorescents sequence-specific probes and primers used in the reaction [6]. Many Real-Time PCR assays have been reported, however, most methods are based on a fluorescent dye-labeled TaqMan probe-based system and this system has many advantages [7,8,9]. It is more sensitive than cell-culture techniques, and has a high sensitivity and specificity, when compared to other tests used for diagnosis of the bacteria [4,5]. *Chlamidiacea* comprises a family of bacteria with medical importance and *C. trachomatis* is one of the most important representatives of this family taxonomic. The *C. trachomatis* is the cause of sexually transmitted infection (STI) most prevalent in the world and the sequels of its infection include pelvic inflammatory disease, ectopic pregnancy and infertility [1]. The infection is asymptomatic in 50% of men and 70% of women [2]. According to estimates from the World Health Organization (WHO), 4 millions new cases of infection in the United States, 10 millions in Europe and 92 millions worldwide are reported annually [3]. Several diagnostic methods for *Chlamydial* infection have been tested around the world. There are methods such as cell-culture and non-culture. The culture of *C. trachomatis* is considered as the *gold-standard* method.

Mannose-binding lectin (MBL) is a plasma glycoprotein that is synthesized mainly in the liver capable of binding patterns of carbohydrate molecules predominantly present on the surface of microorganisms (including bacteria, viruses, and fungi) activating the complement system [10]; it can also promote complement-independent opsono-phagocytosis, modulate inflammation, and endorse apoptosis [11,12,13]. Moreover it has recently been shown that MBL can also activate the lectin pathway of complement system by binding to immunoglobulin A (IgA) [14].

MBL deficiency has been shown to be associated with increased susceptibility to many infectious diseases (for review, see [13,17,18]). Several reports suggest that MBL can also modulate the disease severity and can be used as a marker to predict the therapeutic efficacy in some disorders, such as the response to interferon treatment in patients with chronic hepatitis C [19].

Although mutations in the promoter are likely to directly reduce MBL expression by blocking the binding of transcription factors, polymorphisms in the first exon prevent the correct oligomerization of MBL chains and cause the formation of a defective form of the protein [16,17]. Three major mutant alleles in exon 1 of the MBL-2 gene (at codons 52, 54, and 57), as

well as polymorphisms in the promoter region of the gene, have been associated with reduced levels of serum MBL [15].

The present paper analyzed functional polymorphisms in first exon of the MBL-2 gene in women infected by *C. trachomatis* and in a control population no infected by *C. trachomatis* and correlated MBL-2 polymorphisms with host susceptibility to infection by *C. trachomatis*. We hypothesized that mutation in MBL-2 gene could be responsible for different inter-individual immune responses and could explain part of the susceptibility to *C. trachomatis* infection.

METHODS, SUBJECTS, PATIENTS AND SAMPLES

A total of 33 women, positive to *C. trachomatis* infection, were enrolled at the Specialized Ambulatory of Women from the Municipal Township of Recife, Pernambuco State, Brazil. Patients that composed the study group were admitted in this research when found positive for *C. trachomatis,* according the diagnosis made by Real-Time PCR assay. In addition, 99 healthy women with no evidence of *C. trachomatis* infection or who never tested positive for symptomatic *C. trachomatis* were enrolled as healthy control subjects. These subjects were admitted for standard gynecologic control procedures and underwent cervical screening to ensure the absence of *C. trachomatis* infection. All the 132 patients (infected and health) were composed by different population.

This study was conducted directly with samples of vaginal secretion from these patients.The population was composed by all women who sought the health service, with oncotic cytology, presenting or no injuries of low and high degree valid for one year and had not been subject to prior treatment in the uterine cervix in the last six months.

The criteria for inclusion referred to all women who sought the respective healthy service, with cytology smear performed in the networks presented in the municipality or state, or not showing lesions of low and high grade for atypical squamous cells of undetermined significance (ASCUS) cancer of the uterine cervix valid for one year and had not been subject to prior treatment in the uterine cervix in the last six months. However, the exclusion criteria referred to those patients who do not brought cytology smear performed in the networks accredited municipal and/or state valid for one year and/or has undergone treatment in the uterine cervix in the last six months

and/or be patient human immunodeficiency virus (HIV) positive or not accept the conduct anti-HIV.

The significance of the study was explained to the patients, and informed consent was obtained. The study was approved by the Ethical Committee of the Instituto Materno Infantil de Pernambuco and by the Ethical Committee of the Associação Caruaruense de Ensino Superior.

DNA EXTRACTION

Genomic DNA for MBL-2 genotyping was extracted from vaginal secretion using Wizard® Genomic DNA Purification Kit (*Promega Corporation*), from 100 µL of specimens using total nucleic acid solution, according to standard procedures.

PRIMERS

MBL-2 SNP genotyping was performed using the following primers designed with the Primer Express 1.5 software (Applied Biosystems, Foster City, CA, USA): forward primer 5'-AGGCATCAACGGCTTCCCA-3' and reverse primer 5'-CAGAACAGCCCAACACGTACCT-3'. The expected amplicon length is 153 bp and its theoretical melting temperature is 84ºC.

RT-PCR CONDICTIONS

Amplification reactions were performed in a final volume of 25 µL with 1X SYBR Green I Amplification Master Mix (*Applied Biosystem*), 150 picomoles of the forward primer, 50 picomoles of the reverse primer and 10 ng of genomic DNA. The cycling conditions were as follows: 95ºC for 10 min followed by 95ºC for 30 s and 60ºC for 1 min, repeated 45 times in the Rotor Gene-3000 apparatus (Corbett Research Mortlake, Sydney, Australia).

MBL-2 GENOTYPING

At the end of the PCR, the dissociation protocol included a slow heating from 60º to 95ºC in 0.2ºC steps, with an 8-s interval between steps. Melting curve profiles were obtained using the dissociation software of the Rotor Gene- 3000 apparatus. Samples were analyzed in duplicate and

in different PCR runs to test the reproducibility of this technique. In order to confirm the Real-Time PCR assay, all products were run in 1% agarose gel, stained with ethidium bromide.

We genotyped three MBL-2 polymorphisms located in the first exon at codons 52 (rs5030737), 54 (rs1800450), and 57 (rs1800451). These three variant alleles were grouped together in one category (allele 0), because they have a similar functional effect on MBL, blocking a correct formation of oligomers, as indicated by Larsen *et al.* [17], whereas the combination of three wild type alleles were grouped as allele A as suggested by Garred *et al.* [24].

For genotyping of the exon 1 of MBL-2, three samples of DNA extracted from healthy women were previsously sequenced to be used as standards: (i) A/A (homozygous wild gene), (ii) A/0 (heterozygous for gene mutation), and (iii) 0/0 (homozygous for gene mutation). These samples were amplified by thermostat automatic Rotor Gene™ RG 3000 (*Corbett Research-Uniscience, Australia*) and then converted into peak melting temperature assay (Figure 1).

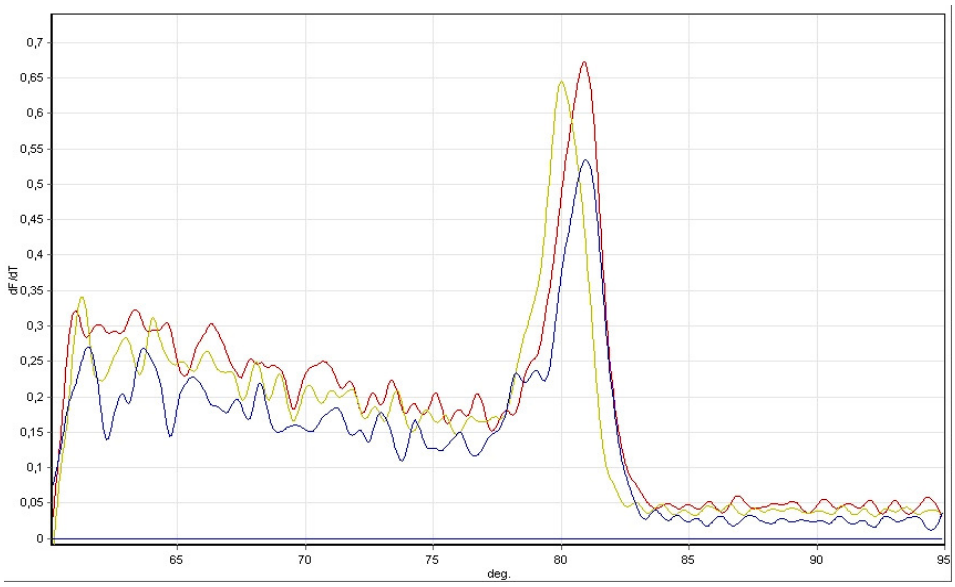

Figure 1. Comparison between the melting curves of the three standards used for genotyping of MBL2. The yellow curve corresponds to the wild type (A/A), in red curve corresponds to the type mutant (0/0), and in blue curve corresponds to the type heterozygous (A/0).

Curves obtained from melting temperature assay can be easily distinguished the three samples amplified. The peak temperature of approximately 83°C corresponds to sample wild (A/A). The

peak temperature of approximately 81°C is the mutant sample (0/0). The sample heterozygote (A/0) for the exon 1 of MBL-2 gene had two peaks, one related to the wild allele and the other related to the mutant allele.

STATISTICAL ANALYSIS

Allele and genotype frequencies were calculated by direct gene counting. *Fisher's* exact test was used for pair wise comparison of genotype frequencies using 2×2 and 3×2 contingency tables and to test for *Hardy-Weinberg* equilibrium.

RESULTS

The results showed that the frequency of the allele A in the study group 64% (22/66) didn't statistically lower than control group 67% (132/198). Furthermore, the allele 0 didn't presented a significantly higher frequency in the infected group 36% (24/66), than control group 23% (46/198) (*p-value* = 0,3112). The Table 1 summarizes the allelic and genotypic frequencies.

Table 1. Comparison between genotypic and allelic frequencies of MBL-2 gene from patients *C. trachomatis* -positive with the healthy control group (*C. trachomatis* -negative).

		Study Group	
Frequencies		*C. trachomatis* (n =33)	Healthy Group (n =99)
Allelic			
	A	0.64 (22/66)	0.67 (132/198)
	0	0.36 (24/66)	0.23 (46/198)
			p= 0.3112
Genotypic	A/A	0,33 (11/33)	0.47 (46/99)
	A/0	0.61 (20/33)	0.40 (40/99)
	0/0	0.06 (2/33)	0,03 (3/99)
			p= 0.1430

[a] *C. trachomatis* -positive *vs* healthy controls (*p-value* = 0.0008577).

Regarding to the genotypic frequencies, the genotype 0/0, in the infected group, didn't showed a significant difference, than in the control group (6% *vs.* 3%, respectively). The genotype A/0 presented a higher frequency in the group infected 61% (20/33) in contrast to the control group 40% (40/99). And the genotype A/A, in the infected group, presented frequency of 33% (11/33), when compared with the control group 47% (46/99), respectively (*p- value* = 0.1430). All samples were within the *Hardy-Weinberg* equilibrium ($\chi^2 = 2.65$).

DISCUSSION

Recent molecular studies showed that there are three possibilities for mutations in the gene exon 1 of MBL-2, which culminates with low or total lack of protein expression [26,27]. The study conducted here, evaluated up the frequency of polymorphisms in the first exon of MBL-2 gene in a cohort of Brazilian women from the State of Pernambuco. The results didn´t showed a significative difference between frequency of the genotype 0/0 in women infected with *C. trachomatis* and healthy group (9% *vs.* 3%, respectively (p = 0.1430). Those results weren't in accordance with others studies which showed that individuals homozygous for mutant alleles have higher risk of contracting infectious disease [23,34,35,36]. Furthermore, these analyses didn´t showed an increase in frequency of the genotype A/A in women in the control group with respect to infected patients (47% *vs.* 33%, respectively).

Since the first report by Super *et al*. (1989) whose authors demonstrated a connection between recurrent infections and low levels of MBL, many papers have been published with regard to an association between MBL deficiency and increased susceptibility to different infections, as well as autoimmune disease [28,29,30]. Nevertheless, it should be mentioned that 90% of MBL-deficient individuals do not suffer from recurrent infections [31]. This can probably be explained by the redundancy of the complement system.

In conclusion, we could say that this study aims at better defining the host immune response, more precisely the innate immunity of MBL, and its role in the *C. trachomatis* infection. Our results indicate that MBL-2 gene polymorphisms didn't seem to be involved in the susceptibility to *C. trachomatis* infection. Therefore, the presence of the mutant allele 0 and genotype 0/0 don´t confer significant risk factors for increased susceptibility to infectious diseases, including

etiology entrusted to *C. trachomatis.* Our data suggest that individuals carrying the functional polymorphisms in the first exon of MBL-2 gene didn´t have correlation with an increased chance of being infected with *C. trachomatis*.

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ANEXOS

ANEXO I

Parecer de Aprovação pelo Comitê de Ética em Pesquisa (CEP)

Comitê de Ética em Pesquisa – CEP/ASCES

CARTA ACP nº. 018/08 CEP/ASCES Caruaru, 30 de Abril de 2008.

Prezado (a) Senhor (a),

Servimo-nos da presente para comunicar-lhe que o protocolo da pesquisa intitulado "POLIMORFISMO NO GENE MBL, EM AMOSTRAS DE SECREÇÃO VAGINAL, DE MULHERES INFECTADAS POR CHLAMYDIA TRACHOMATIS" foi APROVADO após o cumprimento de pendências, conforme deliberação dos membros deste Comitê de Ética em Pesquisa.

Colocamo-nos à disposição para maiores esclarecimentos.

De: Edmilson Maciel Jr.
Coordenador do CEP/ASCES

Para: Ana Catarina Simonetti
Professor da ASCES

Av. Portugal, nº 584 Bairro Universitário Caruaru Pernambuco
Telefone.: 081-2103-2000 Fax.: 081-2103-2014
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AUTORIZAÇÃO PARA REALIZAÇÃO DA PESQUISA

O presente projeto foi devidamente analisado pela Coordenação do Ambulatório Especializado da Mulher da Prefeitura Municipal do Recife , não havendo quaisquer objeções nem entraves administrativos à sua realização.

Recife, 14 de julho de 2006.

U. M. 129
Georgina de O. Campelo
Gerente
Mat. 41.397-0

Georgina de Oliveira Campelo

Diretora do Ambulatório Especializado da Mulher

DECLARAÇÃO

Eu, **ANA CRISTINA REIS BEZERRA**, Diretora do Laboratório Municipal de Saúde Pública da Prefeitura do Recife, em conjunto com a pesquisadora mestranda, **MICHELINE OLIVEIRA LOBO PEREIRA DA COSTA**, abaixo assinados, com o projeto de tese : FREQÜÊNCIA DA INFECÇÃO DA CHLAMYDIA TRACHOMATIS EM PACIENTES COM E SEM LESÕES PRECURSORAS DO CÂNCER CERVICAL ATENDIDAS NO AMBULATÓRIO ESPECIALIZADO DA MULHER DA PREFEITURA MUNICIPAL DO RECIFE, comprometemo-nos a cumprir os termos da Resolução no. 196 de 10.10.1997, do Conselho Nacional de Saúde, conforme o informe epidemiológico do SUS-Brasil, Ano V, no. 2, 1996

Recife, 15 de julho de 2006.

Diretora do Laboratório Municipal de Saúde Pública da Prefeitura do Recife

Pesquisadora

ANEXO II

Normas da Revista Human Immunology

HUMAN IMMUNOLOGY

Official Journal of the American Society for
Histocompatibility and Immunogenetics
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Guide for Authors

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Journal. Leen MPJM, Gorski J. Differential expression of isomorphic HLA-DR genes is not a sole function of transcription. Hum Immunol 1996;50:111-15.
Book. Peter JB, Shoenfeld Y. Autoantibodies. Amsterdam: Elsevier; 1996.
Chapter in a book. Bendtzen K, Hansen MB, Ross C, Svenson M. Cytokine autoantibodies. In: Peter JB, Shoenfeld Y, editors. Autoantibodies. Amsterdam: Elsevier; 1996.
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ANEXO III

Confimação de Submissão do Artigo n°01 para Revista Human Immunology

Subject: Submission Confirmation
Date: quarta-feira, 23 de julho de 2008 09:53
From: Human Immunology <js1040@columbia.edu>
To: Danyelly Bruneska <bruneska@prospecmol.org>

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Human Immunology

ANEXO IV

Normas da Revista Journal of Clinical Microbiology

JOURNAL OF CLINICAL MICROBIOLOGY
2008 INSTRUCTIONS TO AUTHORS*
SCOPE

The *Journal of Clinical Microbiology* (JCM) is devoted to the dissemination of new knowledge concerning the microbiological aspects of human and animal infections and infestations, particularly their etiological agents, diagnosis, and epidemiology. **Case Reports will be considered if they are novel, add to existing knowledge, and are oriented toward microbiology.** (See p. 12 for a description of the two different types of Case Reports published.) Manuscripts describing members of the ''normal'' human microbiota that become involved in disease production or complication and manuscripts dealing with the interactions of hospitalized patients and the microbial environment of the hospital may also be submitted for consideration.

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2 2008 JCM INSTRUCTIONS TO AUTHORS J. CLIN. MICROBIOL.

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collection, transport, and processing: virology, p. 1227–1241. *In* P. R. Murray, E. J. Baron, M. A. Pfaller, J. H. Jorgensen, and R. H. Yolken (ed.), Manual of clinical microbiology, 8th ed. ASM Press, Washington, DC.
8. **Garcia, C. O., S. Paira, R. Burgos, J. Molina, J. F. Molina, and C. Calvo.** 1996. Detection of salmonella DNA in synovial membrane and synovial fluid from Latin American patients. Arthritis Rheum. **39**(Suppl.)**:**S185. {*Meeting abstract published in journal
supplement.*}
9. **Green, P. N., D. Hood, and C. S. Dow.** 1984. Taxonomic
status of some methylotrophic bacteria, p.

251–254. *In* R. L. Crawford and R. S. Hanson (ed.), Microbial growth on C1 compounds. Proceedings of the 4th International Symposium. American Society for Microbiology, Washington, DC.
10. **Odell, J. C.** April 1970. Process for batch culturing. U.S. patent 484,363,770. {*Include the name of the patented item/process if possible; the patent number is mandatory.*}
10 2008 JCM INSTRUCTIONS TO AUTHORS J. CLIN. MICROBIOL.
11. **O'Malley, D. R.** 1998. Ph.D. thesis. University of California, Los Angeles. {*Title is optional.*}
12. **Rotimi, V. O., N. O. Salako, E. M. Mohaddas, and L. P. Philip.** 2005. Abstr. 45th Intersci. Conf. Antimicrob. Agents Chemother., abstr. D-1658. {*Abstract title is optional.*}
13. **Smith, D., C. Johnson, M. Maier, and J. J. Maurer.** 2005. Distribution of fimbrial, phage and plasmid associated virulence genes among poultry *Salmonella enterica* serovars, abstr. P-038, p. 445. Abstr. 105th Gen. Meet. Am. Soc. Microbiol. American Society for Microbiology, Washington, DC. {*Abstract title is optional.*}
14. **Stratagene.** 2006. Yeast DNA isolation system: instruction manual. Stratagene, La Jolla, CA. {*Use the company name as the author if none is provided for a company publication.*}
*A reference to an in-press ASM publication should state the control number (e.g., JCM00577-08) if it is a journal article or the name of the publication if it is a book.
Online references must provide the same information that print references do, but some variation is allowed. For online journal articles, posting or revision dates may replace the year of publication, and a DOI or URL may be provided in addition to or in lieu of volume and page numbers. Some examples follow.
1. **Charlier, D., and N. Glansdorff**. September 2004, posting date. Chapter 3.6.1.10, Biosynthesis of arginine and polyamines. *In* R. Curtiss III et al. (ed.), EcoSal—*Escherichia coli* and *Salmonella*: cellular and molecular biology. ASM Press, Washington, DC. http://www.ecosal.org/ecosal/index.jsp. {*Note that each chapter has its own posting date.*}
2. **Dionne, M. S., and D. S. Schneider.** 2002. Screening the fruitfly immune system. Genome Biol. **3:** REVIEWS1010. http://genomebiology.com/2002/3/4/reviews/1010.
3. **Smith, F. X., H. J. Merianos, A. T. Brunger, and D. M. Engelman.** 2001. Polar residues drive association of polyleucine transmembrane helices. Proc. Natl. Acad. Sci. USA **98:**2250–2255. doi:10.1073/pnas.041593698.
4. **Winnick, S., D. O. Lucas, A. L. Hartman, and D. Toll.** 2005. How do you improve compliance? Pediatrics **115:**e718–e724.
NOTE: A posting or accession date is required for any online reference that is periodically updated or changed.
(ii) References cited in the text. References to unpublished data, manuscripts submitted for publication, unpublished conference presentations (e.g., a report or poster that has not appeared in published conference proceedings), personal communications, patent applications and patents pending, computer software, databases, and websites (home pages) should be made parenthetically in the text as follows.
. . . similar results (R. B. Layton and C. C. Weathers, unpublished data).
. . . system was used (J. L. McInerney, A. F. Holden, and P. N. Brighton, submitted for publication).
. . . as described previously (M. G. Gordon and F. L. Rattner, presented at the Fourth Symposium on Food Microbiology, Overton, IL, 13 to 15 June 1989). {*For nonpublished abstracts, posters, etc.*}
. . . this new process (V. R. Smoll, 20 June 1999, Australian Patent Office). {*For non-U.S. patent applications, give the date of publication of the application.*}
. . . available in the GenBank database (http://www.ncbi.nlm.nih.gov/Genbank/index.html).
. . . using ABC software (version 2.2; Department of Microbiology, State University [http://www.stu.micro]).
URLs for companies that produce any of the products mentioned in your study or for products being sold may NOT be included in the article. However, company URLs that permit access to scientific data related to the study or to shareware used in the study are permitted.
(iii) References related to supplemental material. References that are related **only** to supplemental material hosted by ASM or posted on a personal/institutional website should not be listed in the References section of an article; include them with the supplemental material itself.
(iv) Referencing publish-ahead-of-print manuscripts. Citations of ASM Accepts manuscripts should look like

the following example.
Wang, G. G., M. P. Pasillas, and M. P. Kamps. 15 May 2006. Persistent transactivation by Meis1 replaces Hox function in myeloid leukemogenesis models: evidence
for co-occupancy of Meis1-Pbx and Hox-Pbx complexes
on promoters of leukemia-associated genes. Mol. Cell. Biol. doi:10.1128/MCB.00586-06.
If an author of an article cites an ASM Accepts manuscript
in his paper but wishes at the proof stage to
change the reference entry to that for the published article, the following style should be used:
Wang, G. G., M. P. Pasillas, and M. P. Kamps. 15 May 2006. Persistent transactivation by Meis1 replaces Hox function in myeloid leukemogenesis models: evidence for co-occupancy of Meis1-Pbx and Hox-Pbx complexes on promoters of leukemia-associated genes. Mol. Cell. Biol. doi:10.1128/MCB.00586-06. (Subsequently published, Mol. Cell. Biol. **26:**3902–3916, 2006.)
Other journals may use different styles for their publish-ahead-of-print manuscripts, but citation entries
must include the following information: author name(s), posting date, title, journal title, and volume and page

numbers and/or DOI. The following is an example:
Zhou, F. X., H. J. Merianos, A. T. Brunger, and D. M.
Engelman. 13 February 2001, posting date. Polar residues
drive association of polyleucine transmembrane helices. Proc. Natl. Acad. Sci. USA doi:10.1073/pnas.041593698.

Notes

The Note format is intended for the presentation of brief observations that do not warrant full-length papers. However, Notes should contain firm data; observations alone are not acceptable. Submit Notes in the same way as full-length papers. *They receive the same review, they*
are not published more rapidly than full-length papers, and
they are not considered preliminary communications.
Each Note must have an **abstract of no more than 50 words**. Do not use section headings in the body of the Note; combine methods, results, and discussion in a single
section. Paragraph lead-ins are permissible. The text should be kept to a minimum and if possible **should not exceed 1,000 words**; the number of figures and tables should also be kept to a minimum. **Materials and methods**
should be described in the text, not in figure legends or table footnotes. Acknowledgments should be presented
as in full-length papers, but no separate heading is used. The References section is identical to that of full-length papers.

Minireviews

Minireviews are expected to be focused discussions of defined topics relevant to clinical microbiologists. In general, they are to be submitted only after invitation by one of the JCM editors. Unsolicited Minireviews are discouraged. The cover letter should state whether the article was solicited and by whom.
Minireviews are not expected to be comprehensive reviews of the literature but rather very directed discussions
of specific issues, with emphasis on the views of the author(s). Thus, they may not exceed 12 double-spaced manuscript pages in length, inclusive of illustrations, tables,
and references. References should be limited to 20 or fewer. Minireviews do not have abstracts. In the Abstract
section of the submission form, put "Not Applicable." The body of the Minireview may either have
section headings or be set up like a Note (see above). Minireviews should be submitted via Rapid Review. Minireviews will be reviewed by two JCM editors, with the aim of expedited processing. In general, it is hoped that, barring the necessity of major revisions, accepted Minireviews will appear in print within 3 months of their
submission.

Guest Commentaries

Guest Commentaries are *invited* communications concerning
relevant topics in clinical microbiology that are not necessarily covered by Minireviews. They are intended
to engender discussion and stimulate consensus statements by such organizations as the American Academy
of Microbiology, Clinical and Laboratory Standards Institute, etc. Reviews of the literature, methods and other how-to papers, and responses targeted at a specific published paper are not appropriate. Guest Commentaries
are subject to review.
The length may not exceed 4 printed pages, and the format is like that of a Minireview (see above). Commentaries
should be submitted via Rapid Review.

Case Reports

While a full-length article or a Note may contain a case report section when the report is incidental to the rest of the paper, a specific Case Report format must be used when the report constitutes the entire article.

A Case Report must include an abstract of no more than 50 words. The text starts with presentation of the case under the section heading "Case Report"; there is *no* introductory text before the Case Report heading. After the case is presented, the rest of the text follows in a separate section after a ruled line to separate the sections. No separate head is used for this short discussion
section, but paragraph lead-ins are permitted. The total number of tables and figures (combined) must not exceed 3. For an example of a correctly formatted Case Report, see J. Clin. Microbiol. **39:**1678–1679, 2001.

Letters to the Editor

Two types of Letters to the Editor may be submitted. The first type (Comment Letter) is intended for comments
on final, typeset articles published in the journal (*not* on publish-ahead-of-print manuscripts) and must cite published references to support the writer's argument.

The second type (New-Data Letter) may report new, concise findings that are not appropriate for publication
as full-length papers or Notes.

Letters may be **no more than 500 words long and must**
be typed double spaced. Refer to a recently published Letter for correct formatting. Note that authors and affiliations are listed at the foot of the Letter. Provide only the primary affiliation for each author.

All Letters to the Editor must be submitted electronically,
and the type of Letter (New Data or Comment) must be selected from the drop-down list in the submission
form. For Letters commenting on published articles, the cover letter should state the volume and issue in which the article was published, the title of the article, and the last name of the first author. In the Abstract section of the submission form, put "Not applicable." Letters to the Editor do not have abstracts. Both types of Letter must have a title, which must appear on the manuscript and on the submission form. Figures and tables should be kept to a minimum.

If the Letter is related to a published article, it will be sent to the editor who handled the article in question. If the editor believes that publication is warranted, he will solicit a reply from the corresponding author of the article and give approval for publication.

New-Data Letters will be assigned to an editor accord-

ing to subject matter and will be reviewed by that editor and/or a reviewer.

Please note that some indexing/abstracting services do not include Letters to the Editor in their databases.

Fast-Track Communications

The fast-track route is intended for accelerated review of *short* communications that are of *significant* interest to
clinical microbiologists. Manuscripts are limited to 750 words, one figure, one table, and 10 or fewer references. The format should be the same as that of a new-data letter (see Letters to the Editor, above). Fast-track articles
should be submitted via Rapid Review.

A fast-track submission is subject to approval as such by the editor in chief. If approved for the fast-track route, the manuscript will be assigned to an appropriate JCM editor and reviewed, according to the same standards
applied for traditional manuscripts, within 1 week.

If accepted, the manuscript will be scheduled for the next available issue and edited. An acceptance letter and copyright agreement will be mailed to the corresponding
author. Proofs will be made available electronically as for regular articles.

A fast-track submission that is not approved for the fast-track route will be handled as a New-Data Letter according to normal procedures.

Errata

The Erratum section provides a means of correcting errors that occurred during the writing, typing, editing, or printing (e.g., a misspelling, a dropped word or line, or mislabeling in a figure) of a published article. Submit Errata via Rapid Review (see "How To Submit Manuscripts,"
above). In the Abstract section of the submission form (a required field), put "Not Applicable." Upload the text of your Erratum as an MS Word file. Please see a recent issue for correct formatting.

Authors' Corrections

The Author's Correction section provides a means of correcting errors of omission (e.g., author names or citations)
and errors of a scientific nature that do not alter the overall basic results or conclusions of a published article
(e.g., an incorrect unit of measurement or order of magnitude
used throughout, contamination of one of numerous cultures, or misidentification of a mutant strain, causing erroneous data for only a portion [noncritical] of the study). *Note that the addition of new data is not permitted.*

For corrections of a scientific nature or issues involving authorship, including contributions and use or ownership
of data and/or materials, all disputing parties must agree, in writing, to publication of the Correction.

For omission of an author's name, letters must be signed
by the authors of the article and the author whose name was omitted. The editor who handled the article will be consulted if necessary.
Submit an Author's Correction via Rapid Review (see "How To Submit Manuscripts," above). In the submission
form, select Erratum as the manuscript type; there is no separate selection in Rapid Review for Authors' Corrections,
but your Correction will be published as such if appropriate. In the Abstract section of the submission form (a required field), put "Not Applicable." Upload the text of your Author's Correction as an MS Word file.
Please see a recent issue for correct formatting. Signed letters of agreement must be supplied as supplemental material (scanned PDF files).

Retractions

Retractions are reserved for major errors or breaches of ethics that, for example, may call into question the source of the data or the validity of the results and conclusions of an article. Submit Retractions via Rapid Review (see "How To Submit Manuscripts," above). In the Abstract section of the submission form (a required field), put "Not Applicable." Upload the text of your Retraction as an MS Word file. Letters of agreement signed by all of the authors must be supplied as supplemental
material (scanned PDF files). The Retraction
will be assigned to the editor in chief of the journal, and the editor who handled the paper and the chairman of the ASM Publications Board will be consulted. If all parties agree to the publication and content of the Retraction,
it will be sent to the Journals Department for publication.

ILLUSTRATIONS AND TABLES

Digital files that are acceptable for production (see below) must be provided for all illustrations on return
of the modified manuscript. (On initial submission, the
entire paper may be submitted in PDF format.)
We strongly recommend that before returning their modified manuscripts, authors check the acceptability of
their digital images for production by running their files
through Rapid Inspector, a tool provided at the following
URL: http://rapidinspector.cadmus.com/RapidInspector /zmw/index.jsp. Rapid Inspector is an easy-to-use Webbased
application that identifies file characteristics that may render the image unusable for production. Illustrations may be continuous-tone images, line drawings, or composites. Color graphics may be submitted,
but the cost of printing in color must be borne by the author. Suggestions about how to reduce costs and ensure
accurate color reproduction are given below.
The preferred format for tables is MS Word; however, WordPerfect and Acrobat PDF are also acceptable (see the section on tables below).

Image Manipulation

Computer-generated images may be processed only minimally. Processing (e.g., changing contrast, brightness,
or color balance) is acceptable only if applied to all parts of the image, as well as to the controls, equally, and
descriptions of all such adjustments and the tools used
VOL. 46, 2008 2008 JCM INSTRUCTIONS TO AUTHORS 13
(both hardware and software) must be provided in the manuscript. Unprocessed data and files must be retained by the authors and be provided to the editor on request.

Illustrations

File types and formats. As mentioned above, **illustrations**
may be supplied as PDF files for reviewing purposes only on initial submission; in fact, we recommend this option to minimize file upload time. At the modification
stage, production quality digital files must be submitted: TIFF or EPS files from supported applications
or PowerPoint files (black and white only). Except for figures produced in PowerPoint, all graphics submitted
with modified manuscripts must be bitmap, grayscale, or CMYK (*not* RGB). Halftone images (those
with various densities or shades) must be grayscale, *not* bitmap.
Color PowerPoint files are *not* accepted because the application, designed for developing on-screen computer
presentations, uses the RGB color mode whereas the printing process uses the CMYK color mode. Colors that are represented in a PowerPoint image may not be reproducible on a printing press. Although black-andwhite
Microsoft PowerPoint files are accepted, we do *not* recommend use of PowerPoint. PowerPoint requires users
to pay close attention to the fonts used in their images (see the section on fonts below). If instructions for fonts are not followed *exactly*, images prepared for publication are subject to missing characters, improperly

converted characters, or shifting/obscuring of elements or text in the figure. **Use of PowerPoint is therefore not**
recommended for either color or black-and-white illustrations.
Acceptable file types and formats for production are given in the charts above. More-detailed instructions for preparing illustrations are available at http://art.cadmus .com/da/index.jsp. Please review this information before preparing your files. If you require additional information,
please send an e-mail inquiry to digitalart @cadmus.com.
Minimum resolution. It is extremely important that a high enough resolution is used. Any imported images must be at the correct resolution before they are placed. Note, however, that the higher the resolution, the larger the file and the longer the upload time. Publication quality
will *not* be improved by using a resolution higher than the minimum. Minimum resolutions are as follows:
300 dpi for grayscale and color
600 dpi for lettering
1,200 dpi for line art
600 dpi for combination art (lettering and images)
Size. All graphics **MUST be submitted at their intended**
publication size; that is, the image uploaded should be 100% of its print dimensions so that no reduction or enlargement is necessary. Resolution must be at the required level at the submitted size. Include only the significant portion of an illustration. White space must be cropped from the image, and excess space between panel labels and the image must be eliminated.
Maximum width for a 1-column figure: 3 5/16 inches (ca. 8.4 cm)
Maximum width for a 2-column figure: 6 7/8 inches (ca. 17.4 cm)
Minimum width for a 2-column figure: 4 1/4 inches (10.8 cm)
Maximum height: 9 1/16 inches (23.0 cm)

Macintosh

Application File type	Black and white Color (CMYK)[a]
Adobe Illustrator 6.0, 7.0, 8.0, 9.0, 10.0, 11.0 CS	EPS EPS
Adobe InDesign 1.0	EPS EPS
Adobe PageMaker 6.5	EPS EPS
Adobe Photoshop 4.0, 5.0, 5.5, 6.0, 7.0, 8.0 CS	TIFF TIFF
Adobe Photoshop 5.0 LE	TIFF N/A[b]
ChemDraw Pro 5.0	EPS/TIFF EPS/TIFF
Corel Photo-Paint 8.0	TIFF EPS
CorelDRAW 6.0, 8.0	EPS/TIFF EPS
Deneba Canvas 6.0, 7.0, 8.0	EPS/TIFF EPS
Macromedia FreeHand 7.0, 8.0, 9.0	EPS EPS
PowerPoint 98, 2001	PPT[c] N/A[b]
Prism 3 by GraphPad	TIFF N/A[b]
Synergy Kaleidagraph 3.08, 3.51	EPS N/A[b]

[a] Color graphics must be saved and printed in the CMYK mode, *not* RGB.
[b] ASM accepts only black-and-white, not color, graphics created with Kaleidagraph,
Adobe Photoshop 5.0 LE, Prism 3 by GraphPad, and PowerPoint.
[c] For instructions on saving PowerPoint files, refer to the Cadmus digital art website at http://art.cadmus.com/da/index.jsp.

Windows

Application File type	Black and white Color (CMYK)[a]
Adobe Illustrator 7.0, 8.0, 9.0, 10.0, 11.0 CS	EPS EPS
Adobe InDesign 1.0	EPS EPS
Adobe PageMaker 6.5	EPS EPS
Adobe Photoshop 4.0, 5.0, 5.5, 6.0, 7.0, 8.0 CS	TIFF TIFF
Adobe Photoshop 5.0 LE	TIFF N/A[b]
ChemDraw Pro 5.0	EPS/TIFF EPS/TIFF
Corel Photo-Paint 8.0, 9.0	TIFF EPS
CorelDRAW 7.0, 8.0, 9.0	EPS/TIFF EPS
Deneba Canvas 6.0, 7.0	EPS/TIFF EPS
Macromedia FreeHand 7.0, 8.0, 9.0	EPS EPS
PowerPoint 97, 2000, XP	PPT[c] N/A[b]
Prism 3 by GraphPad	TIFF N/A[b]
SigmaPlot 8.01	EPS EPS

[a] Color graphics must be saved and printed in the CMYK mode, *not* RGB.
[b] ASM accepts only black-and-white, not color, graphics created with Adobe Photoshop 5.0 LE, Prism 3 by GraphPad, and PowerPoint.
[c] For instructions on saving PowerPoint files, refer to the Cadmus digital art website at http://art.cadmus.com/da/index.jsp.

14 2008 JCM INSTRUCTIONS TO AUTHORS J. CLIN. MICROBIOL.
Contrast. Illustrations must contain sufficient contrast to withstand the inevitable loss of contrast and detail inherent in the printing process. See also “Color illustrations”
below.
Labeling and assembly. All final lettering, labeling, tooling, etc., **must** be incorporated into the figures. It cannot be added at a later date. If a figure number is included, it **must** appear well outside the boundaries of the image itself. (Numbering may need to be changed at the copyediting stage.) Each figure must be uploaded as a separate file, and any multipanel figures must be assembled
into one file; i.e., rather than uploading a separate file for each panel in a figure, assemble all panels in one piece and supply them as one file.
Fonts. To avoid font problems, set all type in one of the following fonts: Helvetica, Times Roman, European PI, Mathematical PI, or Symbol. All fonts other than these five must be converted to paths (or outlines) in the application with which they were created. For proper font use in PowerPoint images, refer to the Cadmus digital art website, http://art.cadmus.com/da/instructions

/ppt_disclaimer.jsp.
Compression. Images created with Macintosh applications
may be compressed with Stuffit. Images created with Windows applications may be compressed with WinZip or PKZIP.
Color illustrations. *The cost of printing in color must be*
borne by the author. The current color cost per figure may be accessed from the submission form in Rapid Review. For accepted manuscripts, the total cost of the color will be included in the acceptance letter sent out by
ASM. Adherence to the following guidelines, in addition
to the general ones below, will help to minimize costs and to ensure color reproduction that is as accurate as possible.
Because of the requirements of print production, color illustrations **must** be in the CMYK (cyan, magenta,
yellow, black) color space. The "normal" color mode for
most computer software is RGB (red, green, blue), which is also the color space of your computer monitor. Since CMYK is a smaller color space (meaning it can define fewer colors), colors often shift when an RGB file
is converted to CMYK. In particular, figures showing red
or green fluorescence and those with a significant range of colors may be difficult or impossible to reproduce during the printing process.
Color illustrations must be supplied in the CMYK color mode, as either (i) CMYK TIFF images with a resolution of at least 300 pixels per inch (raster files, consisting of pixels) or (ii) Illustrator-compatible EPS files with CMYK color elements (vector files, consisting
of lines, fonts, fills, and images). See the charts above for
a list of supported applications.
We cannot accept any Microsoft Office files (Power-Point, Word, Excel) for color illustrations because they are restricted to the RGB color space.
Drawings
Submit graphs, charts, complicated chemical or mathematical
formulas, diagrams, and other drawings as finished products not requiring additional artwork or typesetting. No part of the graph or drawing may be handwritten. *All* elements, including letters, numbers, and symbols, *must* be easily readable, and both axes of a
graph must be labeled. Keep in mind that the journal is published both in print and online and that the same electronic files submitted by the authors are used to produce both.
When creating line art, please use the following guidelines:
1. **All art MUST be submitted at its intended publication**
size. For acceptable dimensions, see "Size" above.
2. **Avoid using screens (i.e., shading)** in line art. It can be difficult and time-consuming to reproduce these images without moire´ patterns. Various pattern backgrounds
are preferable to screens as long as the patterns are not imported from another application. If you must use images containing screens,
• Generate the image at line screens of 85 lines per inch or lower.
• When applying multiple shades of gray, differentiate the gray levels by at least 20%.
• Never use levels of gray below 20% or above 70% as they will fade out or become totally black upon scanning and reduction.
3. Use thick, solid lines that are no finer than 1 point in thickness.
4. No type should be smaller than 6 points at the final publication size.
5. Avoid layering type directly over shaded or textured areas.
6. Avoid the use of reversed type (white lettering on a black background).
7. Avoid heavy letters, which tend to close up, and unusual
symbols, which the printer may not be able to reproduce in the legend.
8. If colors are used, avoid using similar shades of the same color and avoid very light colors.
In figure ordinate and abscissa scales (as well as table column headings), **avoid the ambiguous use of numbers**
with exponents. Usually, it is preferable to use the appropriate
Syste`me International d'Unite´s (SI) symbols (_ for 10_{-6}, m for 10_{-3}, k for 10_{3}, M for 10_{6}, etc.). A complete listing of SI symbols can be found in the International
Union of Pure and Applied Chemistry (IUPAC) publication *Quantities, Units and Symbols in Physical*
Chemistry (Blackwell Science, Oxford, United Kingdom, 1993); an abbreviated list is available at http: //www.iupac.org/reports/1993/homann/index.html. Thus,
VOL. 46, 2008 2008 JCM INSTRUCTIONS TO AUTHORS 15
a representation of 20,000 cpm on a figure ordinate should be made by the number 20 accompanied by the label kcpm.

When powers of 10 must be used, the journal requires that the exponent power be associated with the number shown. In representing 20,000 cells per ml, the numeral of the ordinate would be ‘‘2’’ and the label would be ‘‘10_4 cells per ml’’ (not ‘‘cells per ml _ 10_{-4}’’). Likewise, an enzyme activity of 0.06 U/ml would be shown as 6 accompanied by the label 10_{-2} U/ml. The preferred designation would be 60 mU/ml (milliunits per milliliter).

Presentation of Nucleic Acid Sequences

Nucleic acid sequences of limited length which are the primary subject of a study may be presented freestyle in the most effective format. Longer nucleic acid sequences must be presented as figures in the following format to conserve space. Print the sequence in lines of approximately 100 to 120 nucleotides in a nonproportional (monospace) font that is easily legible when published with a line length of 6 inches (ca. 15.2 cm). If possible, lines of nucleic acid sequence should be further subdivided into blocks of 10 or 20 nucleotides by spaces within the sequence or by marks above it. Uppercase and lowercase letters may be used to designate the exon-intron structure, transcribed regions, etc., if the lowercase letters remain legible at a 6-inch (ca. 15.2-cm) line length. Number the sequence line by line; place numerals, representing the first base of each line, to the left of the lines. **Minimize spacing between lines of sequence, leaving room only for annotation of the sequence.**

Annotation may include boldface, underlining, brackets, boxes, etc. Encoded amino acid sequences may be presented, if necessary, immediately above or below the first nucleotide of each codon, by using the single-letter amino acid symbols. Comparisons of multiple nucleic acid sequences should conform as nearly as possible to the same format.

Figure Legends

Legends should provide enough information so that the figure is understandable without frequent reference to the text. However, detailed experimental methods must be described in the Materials and Methods section, not in a figure legend. A method that is unique to one of several experiments may be reported in a legend only if the discussion is very brief (one or two sentences). Define all symbols used in the figure and define all abbreviations that are not used in the text.

Tables

Tables that contain artwork, chemical structures, or shading must be submitted as illustrations in an acceptable format at the modification stage. The preferred format for regular tables is MS Word; however, WordPerfect and Acrobat PDF are also acceptable. Note that a straight Excel file is *not* currently an acceptable format. Excel files must be either embedded in a Word or WordPerfect document or converted to PDF *before* being uploaded. **If your modified manuscript contains PDF tables, select “for reviewing purposes only” at the beginning of the file upload process.**

Tables should be formatted as follows. Arrange the data so that **columns of like material read down, not across.** The headings should be sufficiently clear so that the meaning of the data is understandable without reference to the text. See the “Abbreviations” section (p. 18) of these Instructions for those that should be used in tables. Explanatory footnotes are acceptable, but more extensive table “legends” are not. Footnotes should not include detailed descriptions of the experiment. Tables must include enough information to warrant table format; those with fewer than six pieces of data will be incorporated into the text by the copy editor. Table 1 is an example of a well-constructed table.

NOMENCLATURE

Chemical and Biochemical Nomenclature

The recognized authority for the names of chemical compounds is *Chemical Abstracts* (CAS, Columbus, OH) and its indexes. *The Merck Index*, 14th ed. (Merck & Co., Inc., Whitehouse Station, NJ, 2006), is also an excellent source. For biochemical terminology, including abbreviations and symbols, consult *Biochemical Nomenclature and Related Documents* (Portland Press, London, United Kingdom, 1992), available at http://www.chem.qmul.ac.uk/iupac/bibliog/white.html, and the instructions to authors of the *Journal of Biological Chemistry* and the *Archives of Biochemistry and Biophysics* (first issues of each year).

Do not express molecular weight in daltons; molecular weight is a unitless ratio. Molecular mass is expressed in
daltons.
For enzymes, use the recommended (trivial) name assigned by the Nomenclature Committee of the International
Union of Biochemistry (IUB) as described in *Enzyme Nomenclature* (Academic Press, Inc., New York, NY, 1992) and at http://www.chem.qmul.ac.uk/iubmb

TABLE 1. Correlation between detection of V-Z viral antibody by neutralization and by EIA and IAHA*a*

Antibody	No. of samples with V-Z virus-neutralizing antibody	Correlation (%)
	Positive*b*	Negative	
EIA			
Positive	50	4	
Negative	3	64	94
IAHA			
PNoesgiatitvivee*c*	3	176	608	87

a Sera from individuals without evidence of a current V-Z virus infection.
b Titer _ 1:4.
c Titer _ 1:8.

16 2008 JCM INSTRUCTIONS TO AUTHORS J. CLIN. MICROBIOL.

/enzyme/. If a nonrecommended name is used, place the proper (trivial) name in parentheses at first use in the abstract and text. Use the EC number when one has been assigned, and express enzyme activity either in katals (preferred) or in the older system of micromoles per minute.
For nomenclature of restriction enzymes, DNA methyltransferases,
homing endonucleases, and their genes,
refer to the article by Roberts et al. (Nucleic Acids Res. **31:**1805–1812, 2003).

Drugs

Whenever possible, use generic names of drugs; the use of trade names is not permitted.

Nomenclature of Microorganisms

Binary names, consisting of a generic name and a specific epithet (e.g., *Escherichia coli*), must be used for
all microorganisms. Names of categories at or above the genus level may be used alone, but specific and subspecific
epithets may not. A specific epithet must be preceded by a generic name, written out in full the first
time it is used in a paper. Thereafter, the generic name should be abbreviated to the initial capital letter (e.g., *E. coli*), provided there can be no confusion with other genera used in the paper. Names of all taxa (kingdoms, phyla, classes, orders, families, genera, species, and subspecies)
are printed in italics and should be italicized (or underlined) in the manuscript; strain designations and numbers are not. Vernacular (common) names should be in lowercase roman type (e.g., streptococcus, brucella).

For *Salmonella*, genus, species, and subspecies names should be rendered in standard form: *Salmonella*
enterica at first use, *S. enterica* thereafter; *Salmonella*
enterica subsp. *arizonae* at first use, *S. enterica* subsp.
arizonae thereafter. Names of serovars should be in roman
type with the first letter capitalized: *Salmonella*
enterica serovar Typhimurium. After the first use, the serovar may also be given without a species name: *Salmonella*
serovar Typhimurium. For other information regarding serovar designations, see *Antigenic Formulas*
of the Salmonella Serovars, 8th ed. (M. Y. Popoff, WHO
Collaborating Centre for Reference and Research on Salmonella, Institut Pasteur, Paris, France, 2001). For a summary of the current standards for *Salmonella* nomenclature
and the Kaufmann-White criteria, see the
article by Brenner et al. (J. Clin. Microbiol. **38:**2465–2467, 2000), the opinion of the Judicial Commission of the International Committee on Systematics of Prokaryotes
(Int. J. Syst. Evol. Microbiol. **55:**519–520, 2005), and the article by Tindall et al. (Int. J. Syst. Evol. Microbiol.
55:521–524, 2005).
The spelling of bacterial names should follow the *Approved*
Lists of Bacterial Names (Amended) & Index of the Bacterial and Yeast Nomenclatural Changes (V. B. D.
Skerman et al., ed., ASM Press, Washington, DC, 1989) and the validation lists and notification lists published in the *International Journal of Systematic and Evolutionary*
Microbiology (formerly the *International Journal of Systematic*
Bacteriology) since January 1989. In addition, two sites on the World Wide Web list current approved bacterial names: Bacterial Nomenclature Up-to-Date (http://www.dsmz.de/microorganisms/main.php?contentleft_id_14) and List of Prokaryotic Names with Standing in Nomenclature
(http://www.bacterio.cict.fr/). If there is reason to use a name that does not have standing in nomenclature, the name should be enclosed in quotation marks in the title
and at its first use in the abstract and the text and an appropriate statement concerning the nomenclatural status
of the name should be made in the text. "*Candidatus*"

species should always be set in quotation marks.
For guidelines regarding new names and descriptions of new genera and species, see the articles by Tindall (Int. J. Syst. Bacteriol. **49:**1309–1312, 1999) and Stackebrandt
et al. (Int. J. Syst. Evol. Microbiol. **52:**1043–1047, 2002). To validate new names and/or combinations, authors
must submit three copies of their published article to the *International Journal of Systematic and Evolutionary
Microbiology.*
It is recommended that a strain be deposited in at least two recognized culture collections in different countries when that strain is necessary for the description
of a new taxon (Int. J. Syst. Evol. Microbiol. **50:** 2239–2244, 2000).
Since the classification of fungi is not complete, it is the responsibility of the author to determine the accepted
binomial for a given organism. Sources for these names include *The Yeasts: a Taxonomic Study*, 4th ed. (C.
P. Kurtzman and J. W. Fell, ed., Elsevier Science Publishers
B.V., Amsterdam, The Netherlands, 1998), and *Ainsworth and Bisby's Dictionary of the Fungi*, 9th ed.
(P. M. Kirk, P. F. Cannon, J. C. David, and J. A. Stalpers,
ed., CABI Publishing, Wallingford, Oxfordshire, United Kingdom, 2001); see also http://www.speciesfungorum.org/Names/Fundic.asp.
Names used for viruses should be those approved by the International Committee on Taxonomy of Viruses (ICTV) and published in *Virus Taxonomy: Eighth Report
of the International Committee on Taxonomy of Viruses* (C. M. Fauquet et al., ed., Elsevier Academic Press, San Diego, CA, 2005). In addition, the recommendations
of the ICTV regarding the use of species
names should generally be followed: when the entire species is discussed as a taxonomic entity, the species name, like other taxa, is italic and has the first letter and any proper nouns capitalized (e.g., *Tobacco mosaic
virus, Murray Valley encephalitis virus*). When the behavior
or manipulation of individual viruses is discussed, the vernacular
(e.g., tobacco mosaic virus, Murray Valley encephalitis virus) should be used. If desired, synonyms may be added parenthetically when the name is first mentioned.
Approved generic (or group) and family names may also
be used.
Microorganisms, viruses, and plasmids should be given designations consisting of letters and serial numbers. It is generally advisable to include a worker's initials or a descriptive symbol of locale, laboratory, etc., in the
VOL. 46, 2008 2008 JCM INSTRUCTIONS TO AUTHORS 17
designation. Each new strain, mutant, isolate, or derivative
should be given a new (serial) designation. This designation should be distinct from those of the genotype
and phenotype, and italicized genotypic and phenotypic symbols should not be included. Plasmids are named with a lowercase "p" followed by the designation in uppercase letters and numbers. To avoid the use of the same designation as that of a widely used strain or plasmid, check the designation against a publication database
such as Medline.

Genetic Nomenclature

To facilitate accurate communication, **it is important that standard genetic nomenclature be used whenever
possible and that deviations or proposals for new naming
systems be endorsed by an appropriate authoritative body**. Review and/or publication of submitted manuscripts
that contain new or nonstandard nomenclature may be delayed by the editor or the Journals Department
so that they may be reviewed by the Genetics and Genomics Committee of the ASM Publications Board.
**Before submission of manuscripts, authors may direct
questions on genetic nomenclature to the committee's
chairman: Maria Costanzo (e-mail: maria @genome.stanford.edu)**. Such a consultation should be mentioned in the manuscript submission letter.
Bacteria. The genetic properties of bacteria are described
in terms of phenotypes and genotypes. The phenotype describes the observable properties of an organism. The genotype refers to the genetic constitution of an organism, usually in reference to some standard wild type. Use the recommendations of Demerec et al. (Genetics
54:61–64, 1966) as a guide to the use of these terms. If your manuscript contains information including
genetic nomenclature, please refer to the Instructions to Authors in the January issue of the *Journal of Bacteriology*.

‘‘Mutant’’ vs. ‘‘mutation.’’ Keep in mind the distinction
between a *mutation* (an alteration of the primary sequence
of the genetic material) and a *mutant* (a strain carrying one
or more mutations). One may speak about the mapping of
a mutation, but one cannot map a mutant. Likewise, a mutant has no genetic locus, only a phenotype.
“Homology” versus “similarity.” For use of terms that describe relationships between genes, consult the articles
by Theissen (Nature **415:**741, 2002) and Fitch (Trends Genet. **16:**227–231, 2000). “Homology” implies
a relationship between genes that share a common evolutionary
origin; partial homology is not recognized.
When sequence comparisons are discussed, it is more appropriate to use the term “percent sequence similarity”
or “percent sequence identity,” as appropriate.
Tetracycline resistance determinants. The nomenclature
for tetracycline resistance determinants is based on the proposal of Levy et al. (Antimicrob. Agents Chemother.
43:1523–1524, 1999). The style for such determinants is, e.g., Tet B; the space helps distinguish the determinant designation from that for phenotypes and proteins (TetB). The above-referenced article also gives the correct format for genes, proteins, and determinants in this family.
Viruses. The genetic nomenclature for viruses differs from that for bacteria. In most instances, viruses have no
phenotype, since they have no metabolism outside host cells. Therefore, distinctions between phenotype and genotype
cannot be made. Superscripts are used to indicate hybrid genomes. Genetic symbols may be one, two, or three letters.
Eukaryotes. For information about the genetic nomenclature
of eukaryotes, see the Instructions to Authors for *Eukaryotic Cell* and *Molecular and Cellular Biology.*

ABBREVIATIONS AND CONVENTIONS

Verb Tense

ASM strongly recommends that for clarity you use the **past** tense to narrate particular events in the past, including
the procedures, observations, and data of the study that you are reporting. Use the present tense for your own general conclusions, the conclusions of previous
researchers, and generally accepted facts. Thus, most of the abstract, Materials and Methods, and Results will be in the past tense, and most of the introduction and some of the Discussion will be in the present tense.
Be aware that it may be necessary to vary the tense in a single sentence. For example, it is correct to say ‘‘White (30) demonstrat*ed* that XYZ cells *grow* at pH 6.8,’’ ‘‘Figure 2 show*s* that ABC cells fail*ed* to grow at room temperature,’’ and ‘‘Air *was* removed from the chamber and the mice *died*, which *proves* that mice *require*
air.’’ In reporting statistics and calculations, it is correct to say ‘‘The values for the ABC cells *are* statistically
significant, indicating that the drug inhibit*ed*. . . .’’
For an in-depth discussion of tense in scientific writing, see p. 191–193 in *How To Write and Publish a Scientific*
Paper, 6th ed.

Abbreviations

General. Abbreviations should be used as an aid to the reader, rather than as a convenience for the author, and therefore their **use should be limited**. Abbreviations other
than those recommended by the IUPAC-IUB (*Biochemical*
Nomenclature and Related Documents, 1992) should be used only when a case can be made for necessity,
such as in tables and figures.
It is often possible to use pronouns or to paraphrase a long word after its first use (e.g., ‘‘the drug’’ or ‘‘the substrate’’). Standard chemical symbols and trivial names

or their symbols (folate, Ala, Leu, etc.) may also be used.
It is strongly recommended that all abbreviations except those listed below be introduced in the first paragraph in Materials and Methods. Alternatively, define
each abbreviation and introduce it in parentheses the first time it is used; e.g., ‘‘Cultures were grown in Eagle minimal essential medium (MEM).’’ Generally, eliminate
abbreviations that are not used at least three times in the text (including tables and figure legends).
Not requiring introduction. In addition to abbreviations
for Syste`me International d’Unite´s (SI) units of measurement, other common units (e.g., bp, kb, and Da), and chemical symbols for the elements, the following
should be used without definition in the title, abstract, text, figure legends, and tables: DNA (deoxyribonucleic

acid); cDNA (complementary DNA); RNA (ribonucleic acid); cRNA (complementary RNA); RNase (ribonuclease); DNase (deoxyribonuclease); rRNA (ribosomal RNA); mRNA (messenger RNA); tRNA (transfer RNA); AMP, ADP, ATP, dAMP, ddATP, GTP, etc. (for the respective 5_ phosphates of adenosine and other nucleosides) (add 2_-, 3_-, or 5_- when needed for contrast); ATPase, dGTPase, etc. (adenosine triphosphatase, deoxyguanosine triphosphatase, etc.); NAD (nicotinamide adenine dinucleotide); NAD_ (nicotinamide adenine dinucleotide, oxidized); NADH (nicotinamide adenine dinucleotide, reduced); NADP (nicotinamide adenine dinucleotide phosphate); NADPH (nicotinamide adenine dinucleotide phosphate, reduced); NADP_ (nicotinamide adenine dinucleotide phosphate, oxidized); poly(A), poly(dT), etc. (polyadenylic acid, polydeoxythymidylic acid, etc.); oligo(dT), etc. (oligodeoxythymidylic acid, etc.); UV (ultraviolet); PFU (plaque-forming units); CFU (colony-forming units); MIC (minimal inhibitory concentration); Tris [tris(hydroxymethyl) aminomethane]; DEAE (diethylaminoethyl); EDTA (ethylenediaminetetraacetic acid); EGTA [ethylene glycol-bis(_-aminoethyl ether)-*N,N,N_,N_*-tetraacetic acid]; HEPES (*N*-2-hydroxyethylpiperazine-*N*_-2-ethanesulfonic acid); PCR (polymerase chain reaction); and AIDS (acquired immunodeficiency syndrome). Abbreviations for cell lines (e.g., HeLa) also need not be defined. The following abbreviations should be used without definition in tables:

amt (amount) SE (standard error)
approx (approximately) SEM (standard error of the
avg (average) mean)
concn (concentration) sp act (specific activity)
diam (diameter) sp gr (specific gravity)
expt (experiment) temp (temperature)
exptl (experimental) tr (trace)
ht (height) vol (volume)
mo (month) vs (versus)
mol wt (molecular weight) wk (week)
no. (number) wt (weight)
prepn (preparation) yr (year)
SD (standard deviation)

Drugs. Should an author decide to abbreviate the names of antimicrobial agents in a manuscript, the following standard abbreviations are strongly recommended.

Antibacterial agents. Amikacin, AMK; amoxicillin, AMX; amoxicillin-clavulanic acid, AMC; ampicillin, AMP; ampicillin-sulbactam, SAM; azithromycin, AZM; azlocillin, AZL; aztreonam, ATM; carbenicillin, CAR; cefaclor, CEC; cefadroxil, CFR; cefamandole, FAM; cefazolin, CFZ; cefdinir, CDR; cefditoren, CDN; cefepime, FEP; cefetamet, FET; cefixime, CFM; cefmetazole, CMZ; cefonicid, CID; cefoperazone, CFP; cefotaxime, CTX; cefotetan, CTT; cefoxitin, FOX; cefpodoxime, CPD; cefprozil, CPR; ceftazidime, CAZ; ceftibuten, CTB; ceftizoxime, ZOX; ceftriaxone, CRO; cefuroxime (axetil) and cefuroxime (sodium), CXM; cephalexin, LEX; cephalothin, CEF; cephapirin, HAP; cephradine, RAD; chloramphenicol, CHL; cinoxacin, CIN; ciprofloxacin, CIP; clarithromycin, CLR; clinafloxacin, CLX; clindamycin, CLI; daptomycin, DAP; dicloxacillin, DCX; dirithromycin, DTM; doxycycline, DOX; enoxacin, ENX; erythromycin, ERY; fleroxacin, FLE; fosfomycin, FOF; gatifloxacin, GAT; gentamicin, GEN; grepafloxacin, GRX; imipenem, IPM; kanamycin, KAN; levofloxacin, LVX; linezolid, LZD; lomefloxacin, LOM; loracarbef, LOR; meropenem, MEM; methicillin, MET; mezlocillin, MEZ; minocycline, MIN; moxalactam, MOX; moxifloxacin, MXF; nafcillin, NAF; nalidixic acid, NAL; netilmicin, NET; nitrofurantoin, NIT; norfloxacin, NOR; ofloxacin, OFX; oxacillin, OXA; penicillin, PEN; piperacillin, PIP; piperacillin-tazobactam, TZP; quinupristin-dalfopristin (Synercid), Q-D; rifabutin, RFB; rifampin, RIF; rifapentine, RFP; sparfloxacin, SPX; spectinomycin, SPT; streptomycin, STR; teicoplanin, TEC; telithromycin, TEL; tetracycline, TET; ticarcillin, TIC; ticarcillin-clavulanic acid, TIM; tobramycin, TOB; trimethoprim, TMP; trimethoprim-sulfamethoxazole, SXT; trovafloxacin, TVA; and vancomycin, VAN.

_-Lactamase inhibitors. Clavulanic acid, CLA; sulbactam, SUL; and tazobactam, TZB.

Antifungal agents. Amphotericin B, AMB; clotrimazole, CLT; flucytosine, 5FC; fluconazole, FLC; itraconazole, ITC; ketoconazole, KTC; nystatin, NYT; terbinafine, TRB; and voriconazole, VRC.

Antiviral agents. Acyclovir, ACV; cidofovir, CDV; famciclovir, FCV; foscarnet, FOS; ganciclovir, GCV; penciclovir, PCV; valaciclovir, VCV; and zidovudine, AZT.

Reporting Numerical Data

Standard metric units are used for reporting length, weight, and volume. For these units and for molarity, use the prefixes m, _, n, and p for 10_{-3}, 10_{-6}, 10_{-9}, and 10_{-12}, respectively. Likewise, use the prefix k for 10^3. Avoid compound prefixes such as m_ or __. Use _g/ml

VOL. 46, 2008 2008 JCM INSTRUCTIONS TO AUTHORS 19
or _g/g in place of the ambiguous ppm. Units of temperature
are presented as follows: 37°C or 324 K.
When fractions are used to express units such as enzymatic
activities, it is preferable to use whole units, such as ''g'' or ''min,'' in the denominator instead of fractional or
multiple units, such as _g or 10 min. For example, ''pmol/
min'' is preferable to ''nmol/10 min,'' and ''_mol/g'' is preferable
to ''nmol/_g.'' It is also preferable that an unambiguous form such as exponential notation be used;
for example, ''_mol g_{-1} min_{-1}'' is preferable to ''_mol/g/
min.'' Always report numerical data in the appropriate SI
units.
Representation of data as accurate to more than two significant figures must be justified by presentation of appropriate statistical analyses.
For a review of some common errors associated with statistical analyses and reports, plus guidelines on how to
avoid them, see the article by Olsen (Infect. Immun. **71:**6689–6692, 2003).
For a review of basic statistical considerations for virology
experiments, see the article by Richardson and Overbaugh (J. Virol. **79:**669–676, 2005).

Statistics

Statistical analysis of data is a crucial component of scientific publication. Authors who are unsure of proper statistical analysis should have their manuscripts checked by a qualified statistician.
The following is a list of important items that must be considered before manuscript submission. Deficiencies in
any of these areas may delay review and/or publication.
• Statistical analyses were performed on **all** quantitative data regardless of how significant the differences look in the tables or figures.
• Data were appropriately analyzed as parametric (normally distributed) or nonparametric data.
• Parametric and nonparametric data are presented **appropriately**. Means and standard deviations or standard errors are appropriate means of presenting data analyzed by parametric analyses (i.e., *t* test and analysis of variance [ANOVA]), but only medians and surrounding levels (quartiles, quintiles, 10th and 90th percentiles, etc.) are appropriate for nonparametric statistics (Mann-Whitney test, Kruskal-Wallis test, etc.). Means have no meaning in nonparametric analyses.
• For any data in which there are more than **two comparisons** (i.e., between one control and more than one experimental group), an analysis must be done for multigroup comparisons. Such an analysis would usually be an ANOVA for parametric data or a Kruskal-Wallis test for nonparametric data. *t* tests cannot be used when more than two groups are being compared (except as indicated below). Failure to use multigroup tests generates type 1 errors: concluding that two data sets within the overall data set being compared are different when in fact they are not. *Exception: Some statisticians argue that two-group comparisons can be used on multigroup data if the expected outcomes are appropriately*
anticipated before the experiment. For example, data generated by individually testing two unrelated
factors for their effects on a target with only a single, untreated target as a control could be appropriately
analyzed by t tests instead of ANOVA.
• For **all appropriate multigroup comparisons,** two *P* values must be generated and provided in the manuscript. The main *P* value applies to the overall data set and indicates that within that data set at least two groups differ from each other. The overall *P* value does not indicate which two groups are different. The main *P* value and the overall *P* value should be computed by using a post hoc test. For ANOVA, these post hoc tests are usually Dunnett's test (used to compare multiple experimental groups to a single control), the Fisher protected least significant difference (PLSD) test, the Tukey-Kramer test, and the Games-Howell test. Others may be used. Note that each post hoc test has certain underlying assumptions that may not be applicable to the data under analysis. For a Kruskal-Wallis nonparametric ANOVA, the Dunn procedure is appropriate to generate *P* values for two-group comparisons.
• Data presented as endpoints (i.e., LD_{50}, ID_{50}, etc.) contain both the calculated value and a confidence interval with a statistical significance associated with it (95%, 99%, or similar confidence interval), calculated by logit or probit analysis. Simple LD_{50} values such as Reed-Muench calculations may not be used alone.
• When samples are taken multiple times from one experimental entity (i.e., multiple serum samples from one animal, gross pathology scores measured for the same animal over time, growth curves, etc.), one cannot use analyses such as *t* tests, ANOVA, the Mann-Whitney test, etc., because these tests assume that each measure is independent. An entity with a high score on day 1 is more likely to have

a high score on day 2 than is an entity with a low score. It is likely that some expert statistical help will be needed for these situations, usually involving regression analysis, survival analysis, etc.

• **Statistical significance and biological significance are not the same.** There is nothing magic about a *P* value of 0.05. When results from small sample sizes are compared, a *P* value of 0.05 will often be obtained, but it may be dependent on the outcome of a single experimental value. If sample sizes are small, then more-vigorous (i.e., smaller) *P* values may be necessary. If sample sizes are large, *P* values of _0.05 may be important. There should be both statistical and biological significance to the results and conclusions in the manuscript.

For a review of some common errors associated with statistical analyses and reports, plus guidelines on how to
avoid them, see the article by Olsen (Infect. Immun.
20 2008 JCM INSTRUCTIONS TO AUTHORS J. CLIN. MICROBIOL.
71:6689–6692, 2003).

For a review of basic statistical considerations for virology
experiments, see the article by Richardson and Overbaugh (J. Virol. **79:**669–676, 2005).

Isotopically Labeled Compounds

For simple molecules, labeling is indicated in the chemical formula (e.g., $^{14}CO_2$, $^{3}H_2O$, and $H_2{}^{35}SO_4$).

Brackets are not used when the isotopic symbol is attached to the name of a compound that in its natural state does not contain the element (e.g., ^{32}S-ATP) or to a word that is not a specific chemical name (e.g., ^{131}I-labeled protein, ^{14}C-amino acids, and ^{3}H-ligands).

For specific chemicals, the symbol for the isotope introduced
is placed in square brackets directly preceding the part of the name that describes the labeled entity. Note that configuration symbols and modifiers precede the isotopic symbol. The following examples illustrate correct usage:

[^{14}C]urea UDP-[U-^{14}C]glucose
L-[*methyl*-^{14}C]methionine *E. coli* [^{32}P]DNA
[2,3-^{3}H]serine fructose 1,6-[1-^{32}P]bisphosphate
[-^{14}C]lysine [_-^{32}P]ATP

JCM follows the same conventions for isotopic labeling as the *Journal of Biological Chemistry*, and moredetailed
information can be found in the instructions to authors of that journal (first issue of each year).
VOL. 46, 2008 2008 JCM INSTRUCTIONS TO AUTHORS 21