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DIVERSIDADE GENÉTICA DE ISOLADOS DE *Streptococcus pneumoniae* PERTENCENTES A SOROTIPOS NÃO INTEGRANTES DA VACINA 10-VALENTE

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“A microbiologia é fascinante
porque sempre seremos aprendizes...”

Sumiko Ikura Sinto

RESUMO

Streptococcus pneumoniae é um importante patógeno endêmico, associado a infecções com um elevado número de mortes e desfechos desfavoráveis, principalmente em crianças até 5 anos, idosos e pacientes em algum estado de imunocomprometimento, sendo o maior causador de pneumonias adquiridas na comunidade. A introdução das vacinas pneumocócicas, principalmente as conjugadas, apesar de diminuírem a incidência da doença pneumocócica invasiva e sua transmissão, também exerceu uma pressão seletiva sobre a distribuição dos sorotipos circulantes, levando a necessidade de um monitoramento das mudanças ocorridas tanto em termos de colonização quanto de infecções. As técnicas de biologia molecular têm sido cada vez mais utilizadas em monitoramentos epidemiológicos por permitir a caracterização de populações, comparação com amostras pré-existentes, correlação com clones endêmicos internacionais e outras populações. As técnicas de Pulsed-Field Gel Electrophoresis (PFGE) e Multi Locus Sequence Typing (MLST) são as técnicas consideradas padrão-ouro, e como uma técnica alternativa a elas vem ganhando espaço o Multiple-locus Variable Number of Tandem Repeat Analysis (MLVA) na caracterização de isolados de pneumococo. Sendo assim, o objetivo geral do estudo foi avaliar os genótipos de pneumococos dos principais sorotipos que não integram a vacina 10-valente, e o objetivo específico foi avaliar a utilização de um método alternativo para genotipagem de pneumococos (MLVA), comparando-o com os métodos padrão-ouro (PFGE e MLST). Para isso, foram utilizados 87 isolados de pneumococo dos períodos pré (n=23) e pós-vacina (n=64) coletados entre 2007 e 2012, predominantemente de sítios invasivos (n=83). Quanto à distribuição dos sorotipos, 25 amostras pertencem ao sorotipo 3, 15 ao sorotipo 12F, 15 ao sorotipo 20, 13 ao sorotipo 8, 11 ao sorotipo 19A, 5 ao sorotipo 6A e 3 ao sorotipo6C. A média de idade dos pacientes foi de 49,19 ± 23,25 anos, variando de 0 a 85 anos. O MLST foi consistente com PFGE e MLVA,sendo que foram encontradas oito novos STs: três pertencentes ao sorotipo 6A, dois ao sorotipo 19A, um ao 6C, um ao 8 e um ao sorotipo 20. Isolados associados aos clones PMEN Denmark^{12F}-24, Netherlands^{8}-33, Netherlands^{3}-31 e Netherlands^{15B}-37 também foram encontrados. A técnica de MLVA gerou perfis mais discriminatórios, com índice de diversidade de Simpson de 0,986, seguido pelo

PFGE (0,962) e MLST (0,891). A diversidade entre os diferentes sets de MLVA previamente estabelecidos por outros autores demonstrou pequena diferença entre eles, sendo o set de van Cuyck o mais discriminatório (0,975), seguido por Pichon (0,972), Koeck (0,967) e Elberse (0,964). O coeficiente de Wallace demonstrou congruência de 100% da técnica de MLVA em relação a técnica de MLST. A aplicação do MLVA em uma região brasileira ainda não estudada e em sorotipos não vacinais é a principal contribuição deste estudo, técnica que apresenta um elevado poder discriminatório e congruência com as outras técnicas padrão-ouro, com custos reduzidos e facilidade de execução laboratorial.

PALAVRAS-CHAVE: *Streptococcus pneumoniae*, genotipagem, sorotipos não vacinais, MLVA, MLST, PFGE.

ABSTRACT

Streptococcus pneumoniae is an important endemic pathogen, associated with a high number of deaths and other outcomes, especially in children aged less than 5 years, elderly and immunocompromised patients, being the major cause of community-acquired pneumonia. The introduction of pneumococcal vaccines, especially conjugates, despite decreasing the incidence of invasive pneumococcal disease in active, passive and transmission form, also exercised a selective pressure on the distribution of circulating serotypes and associated to disease, leading to the need for a monitoring of changes both in terms of colonization and infections, because some serotypes have become more associated with disease and its incidence has increased in countries where vaccine distribution occurs continuously and homogeneously. Molecular biology techniques have been increasingly used in epidemiological monitoring to allow the characterization of populations, compared with pre-existing isolates, correlation with international clones and other endemic populations. The Pulsed-Field Gel Electrophoresis (PFGE) and Multi Locus Sequence Typing (MLST) techniques are considered the gold standard, and, as an alternative technique, the Multiple-Locus Variable Number of Tandem Repeat Analysis (MLVA) have been increasingly used in characterization of pneumococcal isolates. The general objective of the study was to evaluate the genotypes of pneumococcal serotypes that are not part of the 10-valent vaccine, and the specific objective was to evaluate the use of an alternative method for genotyping of pneumococci (MLVA), comparing it with gold standard methods (PFGE and MLST). For this, 87pneumococcal isolates from the pre (n = 23) and post-vaccination (n = 64) periods collected between 2007 and 2012 were used, predominantly from invasive sites (n = 83). As the distribution of serotypes, 25 samples belong to serotype 3, 15 to serotype 12F, 15 to serotype 20, 13 to serotype 8, 11 to serotype 19A, five serotype 6A and tree to serotype 6C. The mean age of patients was 49.19 ± 23.25 years, ranging from 0 to 85 years. The MLST was consistent with PFGE and MLVA, and eight new STs were found: tree belonging to serotype 6A, two to serotype 19A, one at 6C, one to 8 and one to serotype 20. Isolates associated with PMEN clones Denmark^{12F}-24, Netherlands^{8}-33, Netherlands^{3}-31 and Netherlands^{15B}-37 were also found. MLVA generated more discriminatory profiles with Simpson's diversity index of

0.986, followed by PFGE (0.962) and MLST (0.891). The diversity between the different sets of MLVA previously established by other authors showed little divergence being the van Cuyck's set more discriminatory (0.975), followed by Pichon's (0,972), Koeck's (0.967) and Elberse's (0.964). The coefficient of Wallace showed 100% of congruence of MLVA in relation to MLST. The application of MLVA in a Brazilian region was not studied so far in non-vaccine serotypes, being the main contribution of this study. This technique has a high discriminatory power and consistency with other gold standard techniques, with reduced costs and ease of laboratory performance.

KEYWORDS: *Streptococcus pneumoniae*, genotyping, non-vaccine serotypes, PFGE, MLVA, MLST.

LISTA DE ABREVIATURAS

ACIP	*Advisory Committee on Immunization Practices*
BgaA	β-galactosidase
CDC	*Centers for Disease Control and Prevention*
ChoP	Fosforilcolina
cpsA	Capsular polysaccharide
Eno	Enolase
Hyl	Hialuronidase
LytA	Autolisina
MLST	*Multi Locus Sequence Typing*
MLVA	*Multiple-locus Variable Number of Tandem Repeat Analysis*
NanA	Neuraminidase
OMS	Organização Mundial da Saúde
PavA	Fator pneumocócico A de adesão e virulência
PCR	*Polimerase Chain Reaction*
PCV	*Pneumococcal Conjugate Vaccine*
PFGE	*Pulsed-Field Gel Electrophoresis*
Ply	Pneumolisina
PMEN	*Pneumococcal Molecular Epidemiology Network*
PPSV	*Pneumococcal Polysaccharide Vaccine*
PsaA	Antígeno A de superfície pneumocócica
PspA	Proteína A de superfície pneumocócica
PspC	Proteína ligadora de colina A
SIREVA	Sistema de redes de vigilância de agentes bacterianos responsáveis por pneumonias e meningites
ST	*Sequence Type*
StrH	β-N-acetilglicosaminidase

SUMÁRIO

1. INTRODUÇÃO

1.1. Epidemiologia das infecções pneumocócicas

De seus primeiros relatos até os dias atuais, *Streptococcus pneumoniae* é um patógeno endêmico de preocupação global, devido ao grande número de mortes e outros desfechos desfavoráveis por ele causados, principalmente em crianças até 5 anos, idosos e pacientes em algum estado de imunocomprometimento[1-3].Dados da Organização Mundial da Saúde estimavam que no ano 2000, cerca de 14,5 milhões de episódios de doenças pneumocócicas tenham ocorrido, levando a morte de aproximadamente 826 mil crianças de 1-59 meses, principalmente em países de baixa renda. Uma atualização publicada em 2012, estima que das 8,8 milhões de mortes de crianças menores de 5 anos em todo o mundo no ano de 2008, aproximadamente 541.000 (6%) destas estejam relacionadas a infecções pneumocócicas, em que a pneumonia ainda é a maior causa de morte nesses indivíduos, representando cerca de 485.000 casos (89,6%), seguida de meningites com 38.800 (7,2%) e outras infecções causando 17.400 (3,2%) mortes nessa faixa etária[4].

Considerando que existe uma imensa disparidade entre as diferentes regiões geográficas em termos de desenvolvimento socioeconômico, é interessante avaliar estes dados de forma estratificada para contextualizar de maneira mais acurada o impacto das mortes por doença pneumocócica no mundo. Desta forma podemos indicar dentre as estimativas da OMS, a África como a região mais impactada, sendo responsável por 57,3% do total de óbitos, seguido do Sudeste Asiático com 20%, Mediterrâneo Oriental com 12,7%, Pacífico Ocidental com 6,2%, América Latina com 2,5% e como a região menos atingida, a Europa com 1,3%[5].

O monitoramento epidemiológico de infecções causadas por *S. pneumoniae*, em especial as invasivas, tem sido realizado de maneira constante. Na América Latina, a primeira rede internacional de monitoramento passivo e voluntário foi iniciada em 1993, coordenada pela Organização Pan-Americana de Saúde, com a participação de seis países(Argentina, Brasil, Chile, Colômbia, México e Uruguai), com os objetivos de estabelecer a prevalência dos sorotipos capsulares associados a doença, reforçar a avaliação epidemiológica e laboratorial nesses países e criar

um banco de amostras para caracterizar os sorotipos e avaliar testes diagnósticos. Vinte anos depois, o SIREVA II (sistema de redes de vigilância de agentes bacterianos responsáveis por pneumonias e meningites) inclui além do *S. pneumoniae*, *Neisseria meningitidis* e *Haemophilus influenzae*, realiza também monitoramento de resistência a antimicrobianos, prevalência de clones circulantes e atualmente é constituída por mais de 20 países da América Latina e Caribe[6].

Dados publicados pelo SIREVA II relativos ao ano de 2012 demonstram que entre todas as idades, foram notificados no Brasil 1059 casos de doença pneumocócica invasiva, sendo destes 44,8% casos de meningite, 34,4% bacteremia ou sepse e 19,3% de pneumonia em todas as faixas etárias. A baixa porcentagem de casos de pneumonia em relação aos dados estimados pela OMS pode estar associada ao fato de pneumonias bacterianas não estarem na lista de doenças de notificação compulsória, como ocorre com os casos de meningite, o que poderia levar a subestimação do número de casos[6].

Dados obtidos no banco de dados do Ministério da Saúde (DATASUS) registraram no ano de 2012, 11.092.598 internações hospitalares, sendo destas 681.828 (6,15%) diagnosticadas como pneumonias. Levando em consideração que de acordo com um estudo realizado por Vila-Corcoles no ano de 2009, cerca de 50% das pneumonias adquiridas na comunidade tem como agente etiológico *S. pneumoniae*, este patógeno estaria associado aproximadamente à 3% das internações hospitalares anualmente no país, o que ressalta a sua importância em termos de saúde pública[7, 8].

1.2. *Streptococcus pneumoniae*

Em termos taxonômicos, o *S. pneumoniae* pertence ao filo Firmicutes, classe Bacilli, ordem Lactobacillales, família Streptococcaceae e juntamente a outros estreptococos alfa-hemolíticos, forma um grupo conhecido como grupo viridans o qual pertencem também *Streptococcus mitis*, *Streptococcus oralis*, *Streptococcus cristatus*,*Streptococcus infantis,Streptococcus peroris* e *Streptococcus pseudopneumoniae*[9].

O micro-organismo *S. pneumoniae*, também podendo ser chamado de pneumococo, foi isolado pela primeira vez paralelamente em amostras de saliva, por George Miller Sternberg nos Estados Unidos e por Louis Pasteur na França no ano

de 1880. Seis anos depois, foi relatado por Weichselbaum em Viena, como a principal causa de pneumonias bacterianas adquiridas na comunidade, sendo que na mesma década, esta bactéria também foi associada a outras patologias como meningite, artrite, otite média e endocardite[2, 10, 11].

Atualmente sabe-se que o pneumococo é colonizador natural da região nasofaríngea posterior de humanos, principalmente crianças até 5 anos, consideradas reservatórios deste micro-organismo[1, 4, 12, 13], sendo que pode estar presente também em adolescentes e adultos com frequência decrescente de acordo com o aumento da idade[12, 14]. O estado de portador é considerado um fator predisponente ao desenvolvimento de infecções não invasivas, como otite média e sinusite e também de infecções invasivas, quando há o isolamento de pneumococo em fluidos estéreis, como pneumonia complicada, meningite oubacteremia[13, 15].

Morfologicamente, este micro-organismo apresenta-se na forma de cocos, podendo estar arranjado aos pares com extremidades lanceoladas ou cadeias curtas, possuindo cápsula polissacarídica e uma parede celular clássica de bactérias Gram positivas, medindo de 0,5 a 1,2 µm de diâmetro[16]. São anaeróbios facultativos, não produzem catalase e são nutricionalmente exigentes, tendo seu crescimento favorecido em presença de atmosfera de 5% de CO_2. Após incubação “overnight” em ágar acrescido de 5%sangue de carneiro, suas colônias medem entre 1 e 3 mm de diâmetro, são alfa-hemolíticas e não apresentam pigmentos, podendo apresentar aspecto mucoide, que caracterizam fenotipicamente sorotipos específicos. Incubações prolongadas (entre 24 e 48 horas) podem produzir além de colônias mucoides, colônias com uma depressão central decorrente da ação de autolisinas, que são laboratorialmente conhecidas como morfologia clássica deste micro-organismo, apesar de sempre ser associada a duas provas fenotípicas de confirmação, para diferenciação de *S. pneumoniae* em relação aos outros membros do grupo viridans: suscetibilidade a optoquina e bile-solubilidade[17].

A optoquina (ou cloridrato de etil-hidrocupreína) é um agente antimicrobiano análogo de quinina que teve uso abandonado na terapêutica devido a sua toxicidade ocular, e por provavelmente ser o primeiro antimicrobiano a ter cepas resistentes recuperadas após utilização em tratamento de camundongos em 1912[2]. Esta prova, proposta em 1915 por Moore e Chesney[18]é utilizada diariamente na rotina laboratorial, onde a amostra suspeita de pneumococo é semeada de forma a obter crescimento confluente em ágar sangue e no centro da semeadura se aplica um

disco de 6 mm impregnado com optoquina, que se, após incubação a 37°C em atmosfera contendo 5% CO_2 possuir halo de inibição de crescimento bacteriano ≥14 mm é considerada sensível. Apesar de ser um teste extensamente aplicado, algumas amostras apresentam-se resistentes a esse antimicrobiano, o que pode levar a confusão na identificação deste micro-organismo nos laboratórios e induzir uma terapia antimicrobiana inadequada. Estudos vêm sendo realizados com o intuito de elucidar os mecanismos envolvidos neste fenótipo de resistência, os quais associam mutações pontuais em subunidades da enzima alvo do antimicrobiano, uma H^+-ATPase que é responsável pelo transporte de hidrogênio na célula[19, 20].

O teste de bile-solubilidade é baseado na capacidade das autolisinas do *S. pneumoniae* de degradarem o peptideoglicano como parte da síntese natural da parede celular. Alguns sais biliares, em especial o desoxicolato de sódio em solução a 10%, quando em contato com uma suspensão bacteriana, induz a aceleração da atividade lítica dessas enzimas, destruindo a parede celular da bactéria e levando a diminuição da turbidez da suspensão, já que as células são destruídas e se tornam solúveis[21]. Porém já houve relatos de amostras de pneumococos insolúveis, o que acredita-se estar associado a modificações nos mecanismos regulatórios da atividade das enzimas, ou na alteração da sua estrutura molecular[22, 23].

A identificação laboratorial de *S. pneumoniae* também pode ser realizada por métodos sorológicos e moleculares, que serão adiante pormenorizados.

1.3. Fatores de Virulência

Inúmeros fatores de virulência são responsáveis pela elevada capacidadedo pneumococo de colonizar o trato respiratório e também de causar doença. No processo de colonização, a presença da adesina fosforilcolina (ChoP) facilita a adesão na superfície do epitélio respiratório, enquanto outra adesina chamada proteína ligadora de colina A (PspC) se liga ao receptor polimérico associado ao transporte de imunoglobulina A secretóriae também ao fator H, oferecendo resistência a ação do sistema complemento[12, 24, 25]. Também associada a adesão na célula do hospedeiro, alguns pneumococos podem produzir pili que podem atuar ainda estimulando a produção de citocinas pró-inflamatórias[25]. Outras enzimas como a neuraminidase (NanA), β-galactosidase (BgaA) e β-N-acetilglicosaminidase (StrH)

atuam removendo açúcares terminais de carboidratos do hospedeiro, permitindo a exposição de receptores de aderência que afetam a função de moléculas de eliminação glicosiladas do patógeno e podem também servir como fonte de nutriente[12]. A produção de NanA também pode estar associada a migração para a tuba auditiva no processo de instalação de otite e desempenha papel essencial na formação de biofilme[25].Enzimas como a hialuronidase (Hyl), enolase (Eno) e o fator pneumocócico A de adesão e virulência (PavA) são reconhecidamente importantes no processo de colonização, ligando em componentes do tecido conectivo, plasminogênio e fibronectina, respectivamente, e estão associadas à disseminação, manutenção da carga microbiana e diminuição da sobrevivência do hospedeiro[12].

A modificação do estado de colonizador para patógeno está associada a expressão de inúmeras enzimas e proteínas que estão sendo avaliados como novos alvos para o desenvolvimento de métodos de identificação molecular e novas formulações vacinais. Neste âmbito, convém destacar o papel da pneumolisina (Ply), autolisina (LytA), o antígeno A de superfície pneumocócica (PsaA) e a proteína A de superfície pneumocócica (PspA).

A pneumolisina, codificada pelo gene *ply*, é uma proteína associada a inibição dos movimentos ciliares do epitélios respiratório e neurais, inibição do irrompimento de fagócitos respiratórios, indução da ativação de células T $CD4^+$ e citocinas quimiotáticas e ativando a via clássica do complemento de forma independente da ativação por anticorpos específicos[12, 25].

A autolisina, codificada pelo gene *lytA*, é uma enzima responsável pela degradação do peptideoglicano da parede celular, provocando a lise da célula e liberando assim, proteínas citoplasmáticas que atuam no processo de patogênese, entre elas a pneumolisina[12, 25].

O antígeno A lipoproteico ligante de metal de superfície pneumocócica (PsaA) integra o sistema de transporte ABC que possui especificidade para o manganês, podendo também carregar íons zinco. Esse sistema permite que a bactéria resista ao estresse oxidativo, tanto produzido pelo peróxido de hidrogênio no metabolismo celular, quanto espécies reativas de oxigênio geradas pela resposta imune inata do hospedeiro[12, 25].

A proteína A de superfície pneumocócica (PspA) é uma molécula altamente eletronegativa que impede a ligação do componente C3 do sistema complemento a

superfície celular pneumocócica, inibindo desta forma o processo de opsonização e fagocitose.

Apesar do papel que estes fatores desempenham na instalação e posterior patogênese do micro-organismo, a presença da cápsula polissacarídica é essencial para o sucesso da colonização e das infecções por ele causadas. A produção da cápsula é associada a um único *locus*, o operon *cps*, que possui seus quatro primeiros genes (*cpsA*-D) comuns a praticamente todos os sorotipos, sendo que os demais genes são distintos entre si e responsáveis pela codificação de glicosil transferases, oligossacarídeos e precursores de açúcares ativados. A variável expressão destas enzimas e outros componentes capsulares é responsável pela classificação do pneumococo em 94 sorotipos com composição polissacarídica única para cada um deles[12, 26]. A principal ação da cápsula é anti-fagocitária, protegendo a superfície celular de interações com o sistema imune do hospedeiro e impedindo o processo de opsonofagocitose mediada por componentes do sistema complemento e neutrófilos[27].

A diversidade capsular de *S. pneumoniae* foi primeiramente detectada por reações sorológicas logo após os primeiros relatos do micro-organismo pelos franceses Bezançon e Griffon em 1897, e caracterizadas em tipos1 e 2 por Neufeld e Haendel em 1910. Fred Neufeld, no ano de 1902 descreveu a reação de Quellung (do alemão “inchaço”) na qual se observa o intumescimento capsular devido a modificação do índice de refração, fruto da interação de bactérias encapsuladas com anticorpos anticapsulares específicos. Esta técnica secular, apesar de ser laboriosa, necessitando de profissionais capacitados devido a subjetividade de interpretação dos resultados obtidos, e financeiramente de inviável execução na rotina laboratorial, é realizada em centros de referência sendo padrão ouro para identificação dos sorotipos capsulares[2, 28].

Esforços globais têm sido direcionados para o estabelecimento de técnicas mais acessíveis de sorotipagem de pneumococos. Métodos imunológicos, como aglutinação em látex, ensaio imunoenzimático (ELISA), imunodifusão radial dupla e mais recentemente o sistema de microesferas Luminex apesar de seu custo mais acessível, não possuem especificidade e sensibilidade equivalentes a reação de Quellung para substituí-la. Contudo, com o advento da biologia molecular, métodos baseados na detecção dos genes específicos que codificam os polissacarídeos capsulares dentro do *locus cps*, tem apresentado bons resultados na determinação

do sorotipo de amostras, especialmente após o desenvolvimento de sistemas de reação em cadeia da polimerase (PCR) multiplex direcionados para a detecção dos sorotipos mais associados a infecções pneumocócicas por Pai, Gertz e Beall em 2006, e otimizadas para os sorotipos mais frequentes na América Latina por Dias e colaboradores em 2007[28, 29, 30, 31].

1.4. Imunoprofilaxia

Por sua essencial atuação nos processos de colonização e patogênese das infecções pneumocócicas, a cápsula foi o alvo primário de estudos para desenvolvimento de formulações imunoprofiláticas desde a caracterização por Avery e Heidelberger da natureza polissacarídica dos antígenos tipo específicos na década de 1930, realizada tardiamente em relação a descoberta dos efeitos protetores de antissoros homólogos ao do micro-organismo por Klemperer e Klemperer em 1891[2]. Desde então, estudos foram realizados incessante e frustradamente, até 1944 quando houve sucesso em ensaios clínicos realizados em bases militares americanas, que infelizmente só foram passíveis de desenvolvimento farmacotécnico e distribuição na década de 1970[32].

Os esforços direcionados ao desenvolvimento de formulações vacinais desde os primórdios do conhecimento sobre as infecções pneumocócicas, em especial pneumonias, objetivavam a diminuição dos desfechos desfavoráveis associados as doenças, principalmente óbitos associados a este patógeno. No entanto, na década de 1940, com o advento da produção e distribuição comercial da penicilina, e seu sucesso no tratamento de infecções bacterianas, o interesse pela intervenção preventiva diminuiu, apesar da penicilina não obter a intensidade desejada na diminuição da mortalidade e os primeiros relatos de resistência descritos em modelos animais surgirem na mesma década[2, 10, 33].

A alternativa a estes empecilhos detectados logo após a introdução da terapia antimicrobiana foi ressuscitar a busca de métodos de profilaxia as infecções pneumocócicas, que foi obtida com sucesso no ano de 1977, quando após inúmeros estudos clínicos com resultados promissores realizados principalmente na África, foi licenciada nos Estados Unidos uma vacina contendo 14 polissacarídeos capsulares, e em 1983, com o acréscimo de nove sorotipos (vacina pneumocócica

polissacarídica 23 valente – PPSV23) foram contemplados os sorotipos prevalentes em doenças pneumocócicas em países desenvolvidos[2, 34].

As vacinas polissacarídicas, as primeiras formulações a serem disponibilizadas, são compostas pelos antígenos de 23 polissacarídeos capsulares purificados (pertencentes aos sorotipos 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20A, 22F, 23F, e 33F) e possuem fenol como conservante[11, 34, 35]. A porcentagem de sorotipos isolados causadores de doença contemplados pela PPSV23 é de aproximadamente 90%, porém, o efeito observado é menor, pois os polissacarídeos são pouco imunogênicos, já que desencadeiam resposta humoral dependente de células B e independente de células T, não sendo recomendada pelo CDC para crianças com idade inferior a 2 anos devido a sua baixa geração de memória imunológica[36]. A indicação de uso da PPSV23 inclui pacientes a partir de 6 anos imunocompetentes com doenças cardio-pulmonares e hepáticas crônicas, utilização de implante coclear, diabetes, alcoolistas, fumantes e com fístula liquórica, pacientes com asplenia anatômica ou funcional e imunocomprometidos além de todos os indivíduos acima de 65 anos segundo recomendações do Comitê Consultivo de Práticas de Imunização americano (*Advisory Committee on Immunization Practices*– ACIP). Convém salientar também que a vacina não diminui a carga bacteriana no estado de portador e pode não ser efetiva na prevenção de otite média, manifestação mais frequente de doença pneumocócica[35, 37- 39].

Quando se detectou que as vacinas polissacarídicas não eram imunogênicas o suficiente para exercer um impacto considerável na diminuição da incidência da doença pneumocócica e que os grupos mais atingidos não estavam protegidos, foram desenvolvidas formulações associando os antígenos polissacarídicos a carreadores protéicos, que elevam a imunogenicidade por facilitar o reconhecimento antigênico por linfócitos T-*helper*, gerando memória imunológica de longa duração. A conjugação com proteínas como a $CRM_{197,}$ não tóxica análoga a toxina diftérica, também produz efeito indireto na redução da incidência de doença pneumocócica, pois diminui a carga microbiana do estado de portador[40-43]. As principais indicações das vacinas conjugadas são para lactentes, crianças até cinco anos imunocomprometidas ou com asplenia, também podendo ser utilizada como reforço a pacientes de risco em todas as idades[35, 37, 39].

A primeira vacina pneumocócica conjugada (*pneumococcal conjugate vaccine* – PCV) a ser disponibilizada nos Estados Unidos em 2000 confere imunidade a sete sorotipos (4, 6B, 9V, 14, 18C, 19F e 23F). Diversos estudos têm sido realizados desde então para ampliar o número de sorotipos contemplados pelas formulações conjugadas, como a PCV10 (10 valente – que inclui os sete sorotipos da PCV7 mais os sorotipos 1,5 e 7F) e PCV13 (sorotipos inclusos na vacina PCV10 mais 3, 6A e 19A), disponíveis no mercado a partir de 2009, em substituição da PCV7[5].

No Brasil, a vacina PPSV23 foi exclusivamente disponibilizada para grupos especialmente suscetíveis desde 1992 e em 1999 foi incluída no calendário de vacinação de idosos pertencentes a grupos de risco e institucionalizados. Já em 2001, a PCV7 foi incorporada pelo Ministério da Saúde para atender estes mesmos grupos e comercializada na rede privada, porém não foi distribuída para a população em geral. Em 2010, a PCV10 foi incluída no calendário básico de vacinação e amplamente distribuída na rede pública para crianças a partir de 2 meses a 2 anos sendo aplicada idealmente em um esquema de 3 doses mais um reforço: 2 meses, 4 meses, 6 meses e um ano[44, 45].

Um estudo avaliando o impacto da PCV10 no Brasil demonstrou que, após 2 anos da introdução da vacina no país, a incidência de casos de doença invasiva ocasionada por sorotipos vacinais na população alvo da vacina (crianças menores de 2 anos) diminui significativamente ($p=0,0002$), indicando o efeito benéfico da mesma, apesar de ainda não se observarem reduções significativas em indivíduos maiores de 2 anos, o que pode ser explicado pelo pequeno período pós-vacina avaliado[46], porém é importante ressaltar que o impacto da vacina na incidência de doença e sobre a distribuição dos sorotipos encontra-se em um estado de transição, sendo de essencial importância o monitoramento dessa etapa para avaliar os efeitos dessa intervenção a longo prazo.

Outro estudo realizado na Dinamarca, também demonstra uma diminuição significativa da incidência de doença pneumocócica invasiva, mas em pacientes de todas as idades ($p<0,001$), o que demonstra um efeito de imunidade passiva[47]. Nos Estados Unidos, também se observam diminuições na incidência de meningite pneumocócica em pacientes de todas as idades ($p<0,001$)[48].

1.5. Mecanismos de evasão à vacina

A introdução das vacinas pneumocócicas, principalmente as conjugadas, ao passo que diminuíram drasticamente a incidência da doença pneumocócica invasiva ativa e passivamente (pelo efeito conhecido como imunidade de rebanho) e sua transmissão, também exerceu uma pressão seletiva sobre a distribuição dos sorotipos circulantes e associados a doença, levando a necessidade de um monitoramento das mudanças ocorridas tanto em termos de colonização quanto de infecções[13, 40, 48-51].

O monitoramento epidemiológico na era pré-vacina ofereceu os subsídios necessários as comparações e inferências que puderam ser realizadas no período pós-vacinal, embasando assim as medidas aplicadas na contenção dos fenômenos ocorridos e no entendimento dos mesmos. Três eventos principais de mudança na distribuição dos sorotipos causadores de doença pneumocócica são estudados: o surgimento de novos clones; a expansão de clones pré-existentes, passando a ocupar o nicho ecológico outrora ocupado por sorotipos vacinais ("*serotype replacement"*); e a recombinação genética que altere a expressão do polissacarídeo capsular que previamente pertencia a um sorotipo vacinal, para o fenótipo de um sorotipo não vacinal (*"switching"* capsular). Os dois últimos eventos têm sido relatados em diversos países, principalmente nos Estados Unidos e Europa[52, 53].

Os exemplos mais conhecidos descritos desses eventos estão associados a emergência do sorotipo 19A, como causador de doença invasiva nos Estados Unidos. Através da utilização de técnicas de genotipagem, foi possível estabelecer quais os mecanismos envolvidos no aumento da incidência do sorotipo 19A. A expansão do clone endêmico Netherlands^{15B}-37, caracterizado molecularmente através da técnica de MLST ("*Multi Locus Sequence Typing*") como pertencente a ST199 ("*sequence type*"), é associada desde a era pré-introdução da PCV7 ao sorotipo 19A no país, sendo um exemplo clássico de *replacement*[53]. Outro estudo realizado por Byinton em 2010, observou que além do sorotipo 19A, os sorotipos 1, 3, e 7F também estavam associados a expansão clonal em pacientes pediátricos em Utah[54]. O sorotipo 1, presente na vacina PCV10, demonstrou também ser um exemplo de *replacement*, em amostras brasileiras que por caracterização molecular por PFGE ("*Pulsed-Field Gel Electrophoresis"* – Eletroforese de Campo Pulsado)

estão relacionadas ao clone Sweden[1]-40 que é caracterizada como pertencente a ST304[55]. Mesmo antes da introdução da vacina, observa-se que este fenômeno já ocorria em algumas partes do mundo, fruto de flutuações naturais da distribuição dos sorotipos ou de outras pressões seletivas como, por exemplo, o uso de antimicrobianos[34, 52, 55-57].

O sorotipo 19A também está associado a mecanismos de *switching* capsular. Estudos realizados na Finlândia, Taiwan e Estados Unidos relacionam este sorotipo a eventos de transformação e recombinação genética[53, 58-60]. Na Holanda, eventos de recombinação também foram relatados para os sorotipos 1 e 22F e nos Estados Unidos para os sorotipos 23A e 23B[61, 62].

A ocorrência destes mecanismos de evasão ao efeito protetor das vacinas pneumocócicas tem sido descrita cada vez mais frequentemente em todo o mundo, sendo de crucial importância o monitoramento constante dos sorotipos e características moleculares de *S. pneumoniae*. Apesar da definição do sorotipo ser um dado essencial, atualmente éinsuficiente para designar e compreender como o micro-organismo sobrevive às pressões seletivas as quais ele é submetido. Por isso, técnicas de biologia molecular voltadas para a caracterização genotípica destes isolados vem se tornando uma ferramenta indispensável na detecção de mecanismos de evasão vacinal[53].

A criação de uma rede de vigilância epidemiológica direcionada a caracterização molecular de cepas endêmicas e com perfis de resistência a penicilina e outros antimicrobianos (*"Pneumococcal Molecular Epidemiology Network"* – PMEN) foi criada em 1997 sob a orientação da *"International Union of Microbiological Societies"*, com os objetivos de padronizar a caracterização, nomenclatura e classificação de pneumococos resistentes a agentes antimicrobianos. Utilizando técnicas de biologia molecular como PFGE, BOX-PCR e MLST, atualmente são conhecidos 43 clones disseminados mundialmente[63, 64].

1.6. Emergência de sorotipos não vacinais após a introdução da PCV7

A pressão exercida pela vacina causou a emergência de alguns sorotipos que previamente eram pouco ou não associados a doença. A partir da desocupação do

nicho ecológico dos sorotipos contidos nas formulações imunoprofiláticas, alguns sorotipos começaram a ser mais associados com doença e ter sua incidência aumentada em países onde a distribuição da vacina ocorre de forma continua e homogênea. Exemplos clássicos são os sorotipos 3 e 19A nos Estados Unidos, sendo o 19A associado a empiema e desfechos mais desfavoráveis[54], bem como frequentemente resistente a penicilina juntamente com o sorotipo 6A[46, 65]. Apesar de pouco comum, 6C tem sido associado a casos de meningite no nordeste brasileiro[66]. O Sorotipo 12F possui uma cepa associada a um clone PMEN que é a maior causa de doença em adultos no Japão, meningite na Polônia e surtos no Alasca[67-69]. Outros clones PMEN de relevância pós-vacina pertencem ao sorotipo 8 e aos sorotipos previamente mencionados 3 e 19A. Entre os isolados pertencentes a nossa população, também se destaca o sorotipo 20, porém pouco se sabe sobre sua habilidade de causar doença.

1.7. Técnicas de genotipagem de isolados de *S. pneumoniae*

Em monitoramentos epidemiológicos, a caracterização de populações,comparação com amostras pré-existentes,correlação com clones endêmicos internacionais e outras populações são os principais intuitos a serem alcançados, para que os dados obtidos permitam o estabelecimento de medidas adequadas de diminuição da incidência de doenças e contenção de possíveis surtos. Para atingir esses objetivos, as técnicas de *Pulsed-Field Gel Electrophoresis* e *Multi Locus Sequence Typing* são atualmente consideradas padrão ouro, fornecendo os subsídios necessários para tal[70].

A introdução de novas técnicas tem como principal objetivo suprir as desvantagens apresentadas pelas técnicas padrões e permitir a utilização em um maior número de laboratórios para a obtenção de dados epidemiológicos cada vez mais acurados.

1.7.1. PFGE

A técnica de eletroforese de campo pulsado (“*Pulsed-Field Gel Electrophoresis”* ou PFGE) foi descrita pela primeira vez em 1984 por Schwartz e

Cantor em leveduras[71]. A partir de então, esta técnica é empregada em inúmeros laboratórios para análise do DNA bacteriano na sua totalidade, principalmente em termos de epidemiologia local e caracterização de surtos. Para isso, uma massa bacteriana é emblocada em agarose e submetida à ação de enzimas líticas que permitirão que apenas o cromossomo fique intacto. Após estas etapas, o DNA é submetido à digestão por enzima de restrição por uma enzima de corte raro (no caso do pneumococo, *Sma*I) e os fragmentos por ela gerados são submetidos a eletroforese com alternância de orientação de campo elétrico, que permite a separação de fragmentos de elevado peso molecular (centenas de kilobases até megabases) por forçar estas moléculas a modificar constantemente a direção da migração no gele geram um padrão de bandas específico. As vantagens dessa técnica incluem a boa tipabilidade das amostras, elevado poder discriminatório e reprodutibilidade, porém é laboriosa, exigindo profissionais qualificados para executá-la, dispendiosa e de difícil comparação inter-laboratorial[63, 72, 73].

1.7.2. MLST

A tipagem por sequenciamento de múltiplos *loci* ("*Multi Locus Sequence Typing*" ou MLST) foi primeiramente descrita por Maiden em 1998 e adaptada no mesmo ano para pneumococos por Enright e Spratt[74, 75]. Através da amplificação e posterior sequenciamento de sete genes constitutivos, cada sequência nucleotídica tem um número alélico único, e a combinação dos sete genes gera uma sequência tipo ("*sequence type*" ou ST) que é comparada ao banco de dados existente (disponível em http://www.mlst.net) e novos números alélicos e STs são depositados nesse sítio eletrônico. As principais vantagens desse sistema são, como no PFGE, a boa tipabilidade das amostras, reprodutibilidade e poder discriminatório, tendo a vantagem em relação a técnica anterior, de permitir comparações em nível global das amostras caracterizadas, porém ainda é um processo dispendioso[70, 74, 75].

1.7.3. MLVA

A caracterização genotípica de isolados de *S. pneumoniae* também pode ser realizada através da analise de VNTRs ("*Variable Number of Tandem Repeats*"), que são sequencias curtas de DNA geralmente não codificante, que se repetem em

quantidade variável de forma contígua dentro de um *locus* gênico, sendo utilizada para analisar a diversidade de inúmeros micro-organismos como os patógenos entéricos *Escherichia coli* e *Salmonella enterica*, *Mycobacterium tuberculosis*e *Bacillus anthracis* e *Yersinia pestis*, notórios agentes utilizados em bioterrorismo[76-79].

Utilizando um sistema de PCR uniplex, seguido de eletroforese convencional, é possível detectar o número de repetições associadas ao *locus* de interesse, cuja combinação de vários *loci* gera um perfil que é adicionado a bases de dados com a finalidade de comparação internacional, possuindo um poder discriminatório maior ou comparável e elevada congruência às técnicas previamente descritas, sendo inclusive associada a subtipificação de amostras pertencentes ao mesmo ST, além de fácil execução e ser menos dispendioso e laborioso[70, 80-82].

A maior dificuldade de aplicação do MLVA deve-se a falta de padronização dos *loci* analisados. Um esquema de 18 reações proposto por Koeck em 2005 é atualmente o que possui maior poder discriminatório, que se adiciona a uma base de dados que analisa 17 dos *loci* (http://www.mlva.eu), sendo que outro estudo realizado pelo mesmo grupo descartou a utilização de um *locus* pela dificuldade de amplificação da cepa padrão utilizada como controle[80, 82]. A tentativa de instituição de esquemas que analisem um número menor de *loci* tem sido realizada, no intuito de diminuir ainda mais custos e tempo, porém não há consenso, pois cada combinação de cerca de 7 *loci* é convenientemente direcionada as particularidades de população estudada[70, 81, 82].

1.7.4. Comparação entre as técnicas de genotipagem

As três técnicas previamente descritas são extremamente úteis na comparação de isolados de *Streptococcus pneumoniae*, porém as técnicas padrão-ouro, por sua limitação financeira, são realizadas em sua maioria em laboratórios de pesquisa ou laboratórios de referência em epidemiologia, o que é um fator limitante na obtenção de dados em países com recursos financeiros escassos, onde geralmente a doença pneumocócica é um problema de saúde pública proeminente. Assim, a técnica de MLVA, por ser mais viável economicamente pode servir como uma alternativa para estes casos. Apesar de possuírem alvos diferentes, a congruência entre as três técnicas de acordo com um estudo realizado por Elberse em 2011 com um set de análise de 7*loci*, utilizando o coeficiente de Wallace para

comparação foi de 0,874(IC95%0.825–0.923) para a técnica de MLVA em relação ao MLST e de 0,465 (IC95% 0.396–0.535) em relação ao PFGE[70]. Cabe ressaltar que a dificuldade de estabelecer conjuntos de *loci* padrão devido a ausência de concordância entre os autores que trabalham com esta técnica dificulta a sua disseminação e consolidação, estando ainda aberta a modificações e otimizações. Estudo realizado por Van Cuyck em 2012 demonstra, comparando apenas as técnicas de MLVA com o MLST, não apenas com o esquema padrão composto por 17 *loci* também os esquemas de combinações de *7 loci* propostos por Koeck em 2008, Pichon em 2010, Elberse em 2011, mas também com uma proposiçãodo estudo de um esquema de 7 *loci* universal, coeficientes de correlação de Pearson de 66.8% para o set de 17 *loci*, 59% para o set de Koeck, 65,1% para o set de Pichon, 43,8% para o set de Elberse e 47,2% para o set estabelecido no estudo[70, 81, 82].

1.7.5. Genótipos de pneumococos no Brasil

Os estudos conduzidos no Brasil com a finalidade de caracterização de isolados de *S. pneumoniae* geralmente utilizam a sorotipagem e perfil de suscetibilidade a antimicrobianos para suas avaliações e comparações[46, 83-91]. Quando objetiva-se a analise genotípica desses isolados, recorre-se a técnica de PFGE como principal ferramenta[55, 92]. Porém, como foi previamente mencionado, a comparação entre os laboratórios é uma tarefa árdua e muitas vezes não passível de extrapolação e inferências a partir de dados de outros laboratórios, o que torna o PFGE mais indicado para a análise de surtos locais, perfis semelhantes de suscetibilidade a antimicrobianos ou características ímpares e análise epidemiológica temporal de amostras, realizada no mesmo local[82, 92].

A utilização da técnica de MLST como ferramenta na análise genotípica de isolados de pneumococo vem crescendo no país, porém ainda pouco se sabe sobre as STs prevalentes. Cabe ressaltar a importância de alguns estudos aqui conduzidos, como um realizado na cidade de Salvador por Lamaro-Cardozo em 2012, com o intuito de verificar a partir de um caso de meningite fatal os contatos colonizados do paciente e encontraram o sorotipo 22F pertencente a ST 6403 como sendo o causador do desfecho sendo também encontrado em dois indivíduos[93]. Outro estudo realizado em Salvador avaliou por PFGE e MLST amostras pertencentes ao sorotipo 6C entre amostras de portadores e com doença invasiva[66],

e mais um estudo realizado em favelas da mesma cidade avaliou a colonização utilizando como ferramentas BOX-PCR, PFGE e MLST[94]. No Rio de Janeiro, foi realizado um estudo para avaliar os genótipos de pneumococos isolados em pacientes com meningite apenas utilizando MLST[95]. Em Goiânia, foi realizado um trabalho para avaliar a população de crianças com colonização de nasofaringe também utilizando esta ferramenta[96]. Um estudo contemplando cinco países da América Latina (Argentina, Brasil, Colômbia, México e Uruguai) também teve como ferramenta principal esta técnica para a avaliação de pneumococos sensíveis a penicilina causadores de doença invasiva[97]. Todos os trabalhos previamente citados demonstram a crescente importância desta técnica no contexto epidemiológico brasileiro, porém existe uma restrição geográfica nos trabalhos devido a imensa dimensão territorial do país, já que apenas cidades das regiões Nordeste, Centro-Oeste e Sudeste foram contempladas, sendo que desta forma não se tem disponível características genotípicas dos pneumococos associados tanto a colonização quanto doença na região Sul, que é bastante acometida por este micro-organismo pelo clima mais frio em relação as outras.

Em relação a utilização da técnica de MLVA em estudos brasileiros, não se encontram trabalhos publicados até o momento, ressaltando-se desta forma a necessidade de que mais estudos sejam efetuados para o estabelecimento dos perfis predominantes no país, para que possa atuar como uma técnica alternativa ao MLST, já que todos os estudos realizados no país contaram com parceria de institutos internacionais de pesquisa para sua execução, o que reitera que as limitações desta técnica realmente inviabilizam a sua execução de forma mais disseminada.

2. JUSTIFICATIVA

A importância do monitoramento epidemiológico de isolados de *Streptococcus pneumoniae*tem sido demonstrada ao longo dos anos como crucial no estabelecimento de medidas de diminuição da incidência de doença, desenvolvimento de formulações imunoprofiláticas e controle da disseminação de sorotipos associados a resistência a antimicrobianos e desfechos graves. Desta forma, a caracterização por técnicas de biologia molecular vem se consolidando como ferramenta chave na construção do conhecimento da dinâmica genética desse micro-organismo e facilitando o entendimento de como ele ocorre, especialmente em sorotipos não presentes na PCV10, dada sua emergência associada a mecanismos de evasão vacinal.

3. OBJETIVOS

O objetivo geral do presente estudo foi avaliar os genótipos de pneumococos dos principais sorotipos que não integram a vacina 10-valente.

O objetivo específico do estudo foi:

-- Avaliar a utilização de um método alternativo para genotipagem de pneumococos (MLVA), comparando-o com os métodos padrão-ouro (PFGE e MLST).

4. ARTIGO CIENTÍFICO

Para atingir os objetivos previamente estabelecidos, o texto foi elaborado na forma do manuscrito intitulado "**How MLVA could be an important tool for the characterization of pneumococcal non-vaccine serotypes"** e será submetido apublicação na revista *Journal of Clinical Microbiology,* cujas normas estão apresentadas como anexo 1 deste documento.

HOW MLVA COULD BE AN IMPORTANT TOOL FOR THE CHARACTERIZATION OF PNEUMOCOCCAL NON-VACCINE SEROTYPES

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Abstract

The aim of this study was to evaluate MLVA as an alternative method for the characterization of non-vaccine pneumococcal serotypes. Eighty-seven isolates from the pre (n = 23) and post-vaccination period (n = 64) collected between 2007 and 2012, predominantly from invasive sites (n = 83) were analyzed. Twenty-five isolates belong to serotype 3, 15 to serotype 12F, 15 to serotype 20, 13 to serotype 8, 11 to 19A, 5 to 6A and 3 to 6C. The MLST was consistent with PFGE and MLVA, and eight new STs were found and isolates associated with PMEN clones Denmark^{12F}-24, Netherlands^{8}-33, Netherlands^{3}-31 and Netherlands^{15B}-37 were also found. The MLVA has generated more discriminatory profiles with Simpson's diversity index of 0.986, followed by PFGE (0.962) and MLST (0.891). The diversity among the different sets of previously established MLVA showed little divergence, being Cuyck's set the most discriminatory (0.975), followed by Pichon's (0,972), Koeck's (0.967) and Elberse's (0.964). The coefficient of Wallace showed 100% of congruence between MLVA and MLST. The application of MLVA in a Brazilian region not studied yet and non-vaccine serotypes is the main contributions of the study, with a high discriminatory power and consistency with the gold standard techniques.

Keywords: *Streptococcus pneumoniae*, genotyping, non-vaccine serotypes, PFGE, MLVA, MLST.

Introduction

The epidemiologic relevance of *Streptococcus pneumoniae* is well recognized as its ability to cause severe disease in children and older adults[1-5]. Since the introduction of pneumococcal conjugate vaccines (PCV7, 10, and 13), incidence rates of pneumococcal diseases declined where health care system are effective[6,7]. Despite this positive effect in the decrease of invasive pneumococcal disease (IPD), it is well established that other pneumococcal serotypeswere not covered by vaccines may emerge, occupying the place of vaccine serotypes[8-13].

Despite the well established impact of PCV7 worldwide, few studies have assessed the impact of the introduction of PCV10 in the distribution of serotypes causing disease[6, 7, 14]. This vaccine includes PCV7 serotypes (4, 6B, 9V, 14, 18C, 19F e 23F) plus serotypes 1, 5 and 7F, which had significant prevalence in Brazil in the pre-PCV10 era[15], and was introduced in the public system in Brazil in 2010.

The introduction of molecular biology techniques have brought a great advance in knowledge and understanding of the population dynamics of pneumococcal disease and how it behaves after the introduction of the vaccines, representing a selective pressure. However, such processes are extremely unpredictable, and epidemiological monitoring of these bacteria must be carried out steadily over time[16-20]. Besides serotyping, the main techniques used for evaluation of the impact of introduction of the vaccine are Pulsed-field Gel Electrophoresis (PFGE) and Multi Locus Sequence Typing (MLST). These genotyping tools present high discriminatory power and typeability, being widely used for epimiological purposes. Both PFGE and MLST are, however, costly in technical and financial aspects[21, 22]. In Brazil, few studies used these techniques to analyze the dynamics of

pneumococci and most epidemiologic studies used only serotype and antimicrobial susceptibility profiles to characterize pneumococcal populations[14, 23-27]. The establishment of alternative methods has become necessary, since the countries most impacted by outcomes of pneumococcal disease do not usually have the resources to perform PFGE or MLST.

MLVA has shown promise in the epidemiological evaluation of isolates from patients affected by invasive and non-invasive pneumococcal disease. Applied to the study of population genetic structure of pneumococci belonging to the most frequently found serotypes, this tool shows a good congruence with PFGE and MLST, being cheaper, faster and with higher discriminatory power, leading to subtyping of clones previously characterized[21, 28]. Based on the evaluation of diversity in the number of VNTRs present in non-coding regions of DNA, MLVA has a well-established applicability in the characterization of numerous pathogens such as *Mycobacterium tuberculosis*, *E. coli*, *Salmonella enterica* and bioterrorism agents *Bacillus anthracis* e *Yersinia pestis*[29-32].

The aim of this study was to evaluate MLVA as an alternative for the characterization of pneumococci that are not included among conjugate vaccine serotypes.

Material and Methods

Isolate selection and study design: A previous study from our laboratory defined that serotypes 3, 6A, 6C, 8, 12F, 19A and 20 were the most commonly found among non-vaccine serotypes. We selected representative isolates belonging to these serotypes isolated from patients with invasive and non-invasive disease, in a

period comprising January 2007 to December 2012. Identification was confirmed through conventional tests ascolony morphology, optochin susceptibility and sodium deoxycholatelysis[33]. The isolates were maintained in skim milk with 10% of glycerol in -80°C freezer. A group of 25 isolates belonging to serotype 3, 15 to serotype 20, 15 to 12F, 13 to 8, 11 to 19A, five to 6A and three isolates to serotype 6C was studied. Regarding age, 23 patients had 65 years old or more, eight were aged five years or less and six were aged two years or less; the mean age of patients was 49.19 ± 23.25 (mean ± standard deviation) ranging from 0 to 85.

Serotyping: Serotyping was done by sequencial PCR multiplex[34] and by Quellung reaction using pool, type and factor specific antisera.

DNA Extraction: The DNA extraction was performed following CDC recommendations: one loop of a fresh growth of *S. pneumoniae* was suspended in 200 µL of 5% Chelex® 100 resin solution (Bio-Rad, Hercules, CA, USA) containing 200 µg/mL of Proteinase K (Invitrogen, Life Technologies, Carlsbad, CA, USA). The suspension was incubated at 56°C for 1 h and at 100°C for 10 min. The process was concluded by centrifugation at 12.000 rpm during 3 min, and the supernatant was stored at -20°C.

MLVA: MLVA was performed based on protocol designed by Koeck et al[28]. Briefly, the DNA were submittedto 18 simplex PCR reactions with 10ng of DNA, 200mM of each primer and 45 µL of Platinum® PCR Supermix (Invitrogen, Life Technologies, Carlsbad, CA, USA). Amplifications were done in GeneAmp® PCR System 9700 (Applied Biosystems, Inc, Carlsbad, CA, USA) using the *S. pneumoniae* ATCC BAA-255 (R6) strain as reaction control. The fragment size of each amplicon was analysed by electrophoresis in 2% agarose gel for all loci (with 100bp size ladder - Invitrogen, Life Technologies, Carlsbad, CA, USA) except locus

Spneu 41 and Spneu 42,which were analyzed in 4% agarose gel (with 10bp size ladder – Invitrogen, Life Technologies, Carlsbad, CA, USA), stained with 0,5 µg/mL ethidium bromide, under UV light and photographed on GelDoc™ XR System (Bio-Rad, Hercules, CA, USA). The results obtained were signed with Internal MLVA Type (IMT) composed of a letter (representing the serotype of the sample) followed by a number indicating the specific profile.

MLST: MLST was performed according to Enright & Spratt[35]. Alleles sequences were edited and complementary sense and antisense fragments were aligned using CodonCode Aligner. Allele profile and Sequence Type (ST) were obtained from the database of the MLST web site (www.mlst.net). In cases of new allele or ST, data were submitted for the approval of the curator before been published.

PFGE: PFGE was done according to Pinto et al[36] with some modifications based on McEllistrem and co-workers[37] previously study: a 7h *S. pneumoniae* grown was washed four times with 300µL of PIV (1 M NaCl, 10 mM Tris-HCl [pH 7.6]) before agarose plugs preparation, lysis was performed overnight at 50°C and incubations with ESP solution was performed two times for 2 h.

Data Analysis: MLVA and MLST numerical profiles and PFGE patterns were analysedusing Bionumerics version 7.1 (Applied Maths, Sint-Martens-Latem, Belgium).PFGE patterns were clustered by UPGMA. A dendrogram wasgenerated from a similarity matrix calculated using the Dice similarity coefficient. PFGE clusters were defined as isolates with a similarity of80% or higher on the dendrogram[38]. Clustering of MLST and MLVA types were represented in minimum spanning trees (MST) and clonal complexes (CC) were defined as isolates with a similarity of 70% or higher[28].

The diversity generated by PFGE, MLVA (sets of 18 loci and 7 loci established by van Cuyck in 2012, Koeck 2008, Elberse 2011 and Pichon 2010)[22, 28, 21, 39] and MLS was calculated by Simpson's Index of Diversity (SID) with confidence interval of 95% (95%CI) and congruence was calculated using Wallace coefficient with confidence interval of 95% (95%CI) available on line (http://www.comparingpartitions.info[21]).

Results

In a period of six years, 87 isolates were collected; of which 23 belong to the pre-vaccine period as is shown in Table 1. Most isolates were recovered from invasive sites (n=83), and that the predominant fluid is blood (n=62), followed by cerebrospinal fluid (n=18), pleural fluid (n=2), and peritoneal fluid (n=1). Two isolates were obtained from respiratory specimens. For the remaing two isolates, clinical specimen was not specified. The MLST showed results detailed in Table 2, and consistent with the two other techniques in terms of clustering but less discrimination of the formed groups (Figure 1). Eight new STs were found, three belonging to serotype 6A (ST8879, 8888 and 8892), two to serotype 19A (ST8800 and 8884), ST8887 to the 6C, ST8885 to 8 and ST8889 to serotype 20. It should also be noted that all strains of serotype 12F are ST218, which belongs to the international PMEN clone Denmark^{12F}- 24; the major clonal complex of serotype 8 belonged to ST53, which is assigned to PMEN clone Netherlands^{8}-33. Most of the isolates of serotype 3 belonged to ST180, associated with the PMEN clone Netherlands^{3}-31 and one of the singletons of serotype 19A belonged to ST199, associated with PMEN clone Netherlands^{15B}-37.

The dendrogram generated from PFGE profiles are shown in Figure 2. Seven isolates were not available for PFGE analysis, five belonging to serotype 3 and two to serotype 20. Detailing the analysis of clusters by serotype, PFGE was performed on 20 isolates of serotype 3 (Figure 2A), which were divided into four clonal groups (CG) and two singletons. The main clonal group consisted of six isolates of ST180, followed by one CG composed of four isolates of ST180, one CG consists of three samples belonging to ST 180, one CG composed of three isolates belonging to ST505, one CG with two isolates of ST1116, one singleton of ST180 and another to ST72. Isolates belonging to serotype 6A (Figure 2B), were grouped into one clonal group with isolates belonging to ST5576 and ST8879 and three singletons. Serotype 6C (Figure 2C) presents a CG consisting of three singletons. Serotype 8 (Figure 2D) had only a CG consisting of 11 isolates (ST53) and two singletons. Among isolates of serotype 20 (Figure 2E), all isolates were grouped in only one CG. The strains of serotype 19A were grouped into three singletons and two CG with three isolates (Figure 2F), one CG consists of isolates of ST2878 and the other by three isolates belonging to ST320 (two isolates) and ST8884. All strains of serotype 12F were grouped within the same CG (Figure 2G).

MLVA analysis generated dendrograms consistent with MLST results despite presenting a more discriminatory profile, as represented in the minimum spanning tree in Figure 3 (three isolates of serotype 8 were not recovered). It should be noted that serotype 6A presented two isolates with similar IMT profiles (more than 70% of similarity) and identical PFGE profiles belonging to different STs, representing a SLV. This event also occurs in ST8884 (serotype 19A) that is a SLV of ST320 showing similar IMT profiles and identical PFGE profiles.

The results of Simpson's Index of Diversity are shown in Table 3. The technique that determined a higher genetic diversity, taking into account all serotypes analyzed, was MLVA (0.986), followed by PFGE (0.962) and MLST (0.891). Genetic diversity of different sets of MLVA previously proposed by other authors showed little difference among them, van Cuyck's being the most discriminatory (0.975), followed by Pichon (0.972), Koeck (0.967) and Elberse (0.964).

Wallace coefficient, demonstrating congruence among the results of the techniques is presented in Table 4 where it can be seen that MLVA were 100% congruent with MLST and also showed consistent results PFGE relative to MLST but not as satisfactory.

Discussion

This study characterized genotypically seven non-PCV10 serotypes that were obtained pre- and post-vaccination with PCV10. Previous studies detected a remarkable emergence of serotypes such as 3 and 19A after the introduction of PCV7[40-45]. Other non-vaccine serotypes included 6A, often associated with resistance to penicillin and considered a pediatric serotype[14, 46]; 6C, associated with meningitis cases in northeast Brazil[47]; serotype 12F, associated with a PMEN resistant clones and characterized as a major cause of disease in adults in Japan, meningitis in Polland and outbreaks in Alaska[48-50], and serotype 8 also belonging to a PMEN clone. Little is known about serotype 20 and its ability to cause disease.

PFGE technique is often used in genotype analysis of bacterial isolates, but is the costly and presents difficulties in inter-laboratory comparison, being more appropriate for the analysis of local outbreaks, similar antimicrobial susceptibility

profiles or particularly sample characteristics and temporal epidemiological analysis of isolates obtained in the same location[22, 36, 51].

In Brazil, the vast majority of epidemiological studies aimed to characterize isolates of *S. pneumoniae* based on serotyping and antimicrobial susceptibility profiles[14, 23-27]. MLST is being used with increased frequency, despite its high cost[47, 52-56].

Our data shows that MLVA demonstrated a good level of congruence in relation to previous genotyping studies. In Elberse´s (2011) study in 2011, using the same coefficient for comparison purposes, found a Wallace index of 0.874 whenMLVA was compared to MLST, in our study it was 1.0. Wallace´s index for PFGE compared to MLST was 0.325 in Elberse (2011), and 0.964 in our study. This marked difference, however, may be due to the fact that number of isolates in our study is smaller and a limited number of serotypes has been investigated [21]. A study by Van Cuyck in 2012 also compared the congruence between MLST and MLVA techniques, but we used the Pearson correlation index and obtained congruence of 65.1% which is relatively low compared to the results of our study. Another hypothesis to be considered is that even considering the comparisons made by Van Cuyck in trying to establish a universal panel using seven loci compared to 18 used in our study. The panel of 17 loci obtained a correlation coefficient of 66.8% while the restricted panelestablished in that study had 47.2 % coefficient, compared to 65.1%, 43.8% obtain with other sets of primers[21, 22, 39].

In our study, seven loci panels established by the authors cited above, despite the use of different correlation coefficients for the analysis, proved to be more diverse than the Pearson coefficient of the isolates shown in a study by van Cuyck, which may be related to the particular characteristics of the population studied, isolates

belonging to one of the 10 STs with wider circulation in England and Wales[22]. As the MLST is a technique with less SID than MLVA as demonstrated in our study (SID 0.89) and also supported by the study conducted by Elberse et al. (SID 0.987), this could be a factor that would decrease the diversity of the population characterized by van Cuyck et al.[21,22]. In the study by Pichon and coworkers in 2010, 35 isolates from an outbreak were characterized by MLVA, this set, according to the author, presents a good analysis of diversity by discriminating samples belonging to the same serotype and ST[39],despite the set did not shown a diversity as high as the set of seven loci used by van Cuyck. Elberse's study in 2011 featured 263 carriage isolates between the 1996-2007, achieving a higher SID than that shown in our study (0.993 and 0.964, respectively). Factors that may contribute to the higher genetic diversity are longer study duration, higher sample size and the fact that nasopharyngeal samples are associated with a higher genetic diversity compared to isolates from IPD[21].

Some aspects that may favor the spread of MLVA in a global context include inter-laboratory comparison and portability. Besides this, MLVA does not need sophisticated equipment as PFGE and MLST, making it suitable for less resourced countries. The versatility of using different set of loci provides potential conditions to the characterization by this technique of previously recognized PMEN clones and unification of existing data.

This study has some limitations. Isolates were obtained by passive surveillance of pneumococcal disease, and since PCV10 was recently introduced in Brazil, further studies are necessary for a deeper analysis of the post-vaccine period. Besides this, the number of isolates from children was small in this study, making difficult to establish whether changes in pressure generally suffered from the

introduction of PCV10 as an indirect effect or whether other variables that have not been studied interfered in the process.

The major contribution of our study was to apply MLVA in a selected group of pneumococci from a not previously investigated region and belonging to serotypes not included in PCV-10 vaccine. The techniquehad already established relevance when applied in populations that also included vaccine serotypes; now our data indicate that it may be used in non-vaccines serotypes as well. MLVA presented congruence with other techniques and its high discriminatory power was confirmed in our setting.

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disease in children in five Latin American countries. Microb Drug Resist. **11**(3): 195-204.

Tables

Table 1: Characteristics of the 87 clinical isolates recovered from Southern Brazil analized according their serotypes

Serotype	Number of Isolates	Pre-PCV10	IPD	Age ≤2 years	Age ≤5 years	Age ≥65 years	Average Age±SD
3	25	7	24*	2	3	9	49.87 ± 27.68
6A	5	3	4	1	1	1	39.80 ± 33.14
6C	3	1	2	1	1	0	39.33 ± 32.88
8	13	3	13	1	1	1	43.61 ± 19.32
20	15	4	15	0	0	7	61.27 ± 15.82
12F	15	4	14*	0	0	2	52.71 ± 11.14
19A	11	2	11	1	2	3	40.27 ± 26.92
TOTAL	87	23	83	6	8	23	49.19 ± 23.25

Legend:

IPD Invasive Pneumococcal Disease

SD Standard Deviation

* Data not available for one isolate

Table 2: Distribution of MLST, MLVA and PFGE profiles among evaluated serotypes

Sorotipo	ST(n)	Perfil de MLVA	Perfil de PFGE
3	180 (18)	E1 (2), E2, E3, E4, E5, E6, E7, **E8, E9, E10, E11, E12, E13, E14, E15, E16**, E34	**E1, E3 (2), E4 (2), E5 (2), E6 (2), E7**, E9 (3), E10, NA(4)
	505 (3)	**E28, E29, E30**	**E12, E13 (2)**
	1116 (3)	**E31, E32, E33**	**E14, E15**, NA (1)
	72 (1)	E35	E16
6A	5576 (1)	A5[a]	A4
	1939 (1)	A9	A10
	8879 (1)	A6[a]	A4
	8888 (1)	A10	A1
	8892 (1)	A8	A11
6C	172 (1)	C1	C3
	8887 (1)	C7	C1
	3930 (1)	C5	C6
8	53 (11)	**D1 (4), D2, D3, D4, D5, D6,**NA(2)	**D3 (5), D4, D5 (5)**
	404 (1)	NA	D1
	8885 (1)	D7	D2
20	8889 (15)	**B1 (2), B2 (8), B3, B4, B5, B6, B7**	**B1, B2, B3 (11),**NA(2)
12F	218 (15)	**F1, F2, F3, F4, F5, F6, F7 (2), F8 (4), F9, F10,** F11	F1 (2), F2, F3 (8), F5 (3), F6
19A	2878 (4)	**G1 (2), G2,** G13	**G5 (3)**, G2
	320 (2)	**G8[a], G11[a]**	**G6, G7**
	8800 (1)	G4	G1
	8884 (1)	G10[a]	G6
	733 (1)	G5	G3
	4967 (1)	G3	G9
	199 (1)	G14	G4

Legend:

NA: Data not available

[a]: isolates with ≥70% of similatity between them in the respective serotype

Bold profiles belong to same clonal group according to the technique used.

Table 3: Simpson's Index of Diversity of the different typing methods

Method	N of types	SID	CI (95%)
MLST	23	0.891	(0.857-0.925)
MLVA	62	0.986	(0.973-0.999)
PFGE	40	0.962	(0.941-0.983)
Elberse's MLVA Set[21]	41	0.964	(0.945-0.983)
Pichon's MLVA Set[39]	48	0.972	(0.955-0.988)
Koeck's MLVA Set[28]	41	0.967	(0.951-0.983)
van Cuyck's MLVA Set[22]	45	0.975	(0.961-0.988)

Legend:

SID= Simpson's Index of Diversity

CI=Confidence Interval

Table 4: Congruence between typing methods by Wallace coefficients

	PFGE	MLVA	MLST
PFGE		0.295 (0.107-0.482)	0.964 (0.943-0.985)
MLVA	0.786 (0.684-0.887)		1.000 (1.000-1.000)
MLST	0.339 (0.228-0.449)	0.132 (0.043-0.220)	

Figure Legends

Figure 1: Minimum Spanning Tree generated of analysis of MLST with presence of PMEN clones.

Legend: Grey shadows represents profiles belonging to same clonal group. The size of the circle represents the number of isolates belonging to the same profile.

Figure 2: Dendrogram generated of PFGE profiles of serotypes:

Figure 2A: Serotype 3

Legend: Optimization 0.5% and tolerance 1%

Figure 2B: Serotype 6A

Legend: Optimization 0.5% and tolerance 1%

Figure 2C: Serotype 6C

Legend: Optimization 0.5% and tolerance 1.5%

Figure 2D: Serotype8

Legend: Optimization 0.5% and tolerance 1.5%

Figure 2E: Serotype 20

Legend: Optimization 0.5% and tolerance 1.5%

Figure 2F: Serotype19A

Legend: Optimization 0.5% and tolerance 1%

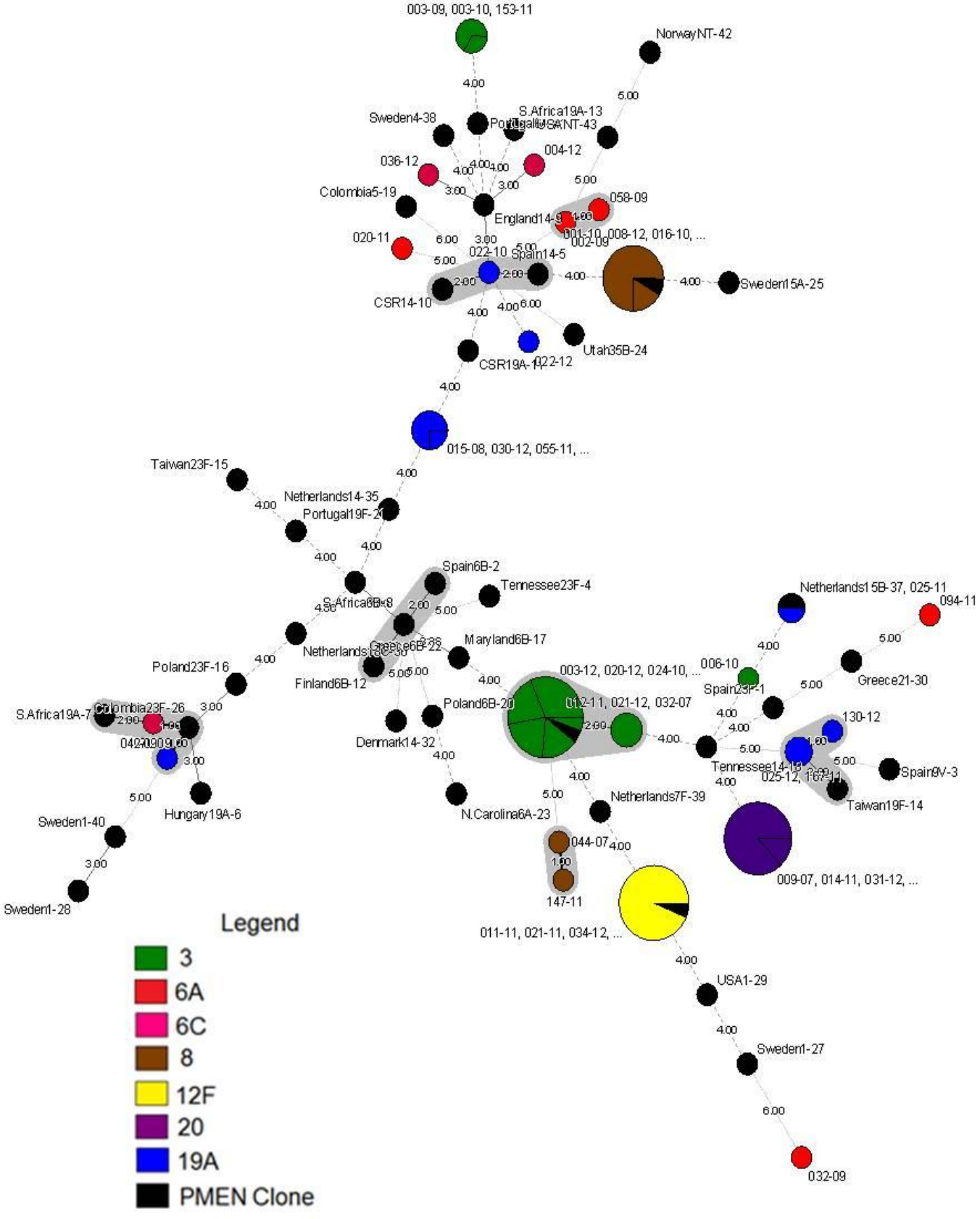
Figure 2G: Serotype 12F

Legend: Optimization 0.5% and tolerance 1.5%

Figure 3: Minimum Spanning Tree generated of analysis of MLVA profiles.

Legend: Grey shadows represents profiles belonging to same clonal group. The size of the circle represents the number of isolates belonging to the same profile.

Figure 1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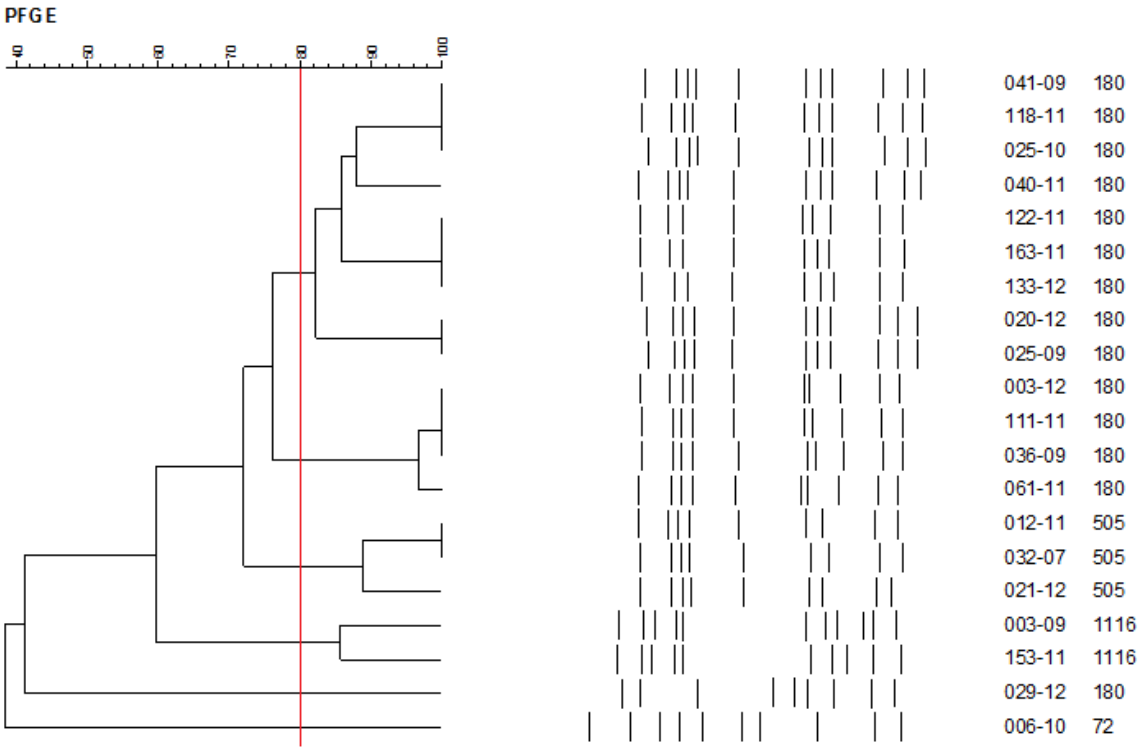
NorwayNT-42
Sweden4-38
S.Africa19A-13
PortugalUSANT-43
004-12
036-12
Colombia5-19
England14-9
058-09
001-10, 008-12, 016-10, ...
002-09
020-11
022-10
Spain14-5
Sweden15A-25
CSR14-10
Utah35B-24
CSR19A-11
022-12
015-08, 030-12, 055-11, ...
Taiwan23F-15
Netherlands14-35
Portugal19F-24
Spain6B-2
Tennessee23F-4
Netherlands15B-37, 025-11
094-11
Maryland6B-17
Greece21-30
006-10
Spain23F-1
003-12, 020-12, 024-10, ...
Poland23F-16
Finland6B-12
012-11, 021-12, 032-07
130-12
S.Africa19A-7
Colombia23F-26
Poland6B-20
Denmark14-32
Tennessee14-18
025-12, 167-11
Spain9V-3
Taiwan19F-14
Hungary19A-6
Sweden1-40
N.Carolina6A-23
Netherlands7F-39
044-07
147-11
009-07, 014-11, 031-12, ...
011-11, 021-11, 034-12, ...
USA1-29
Sweden1-27
Sweden1-28
032-09
Legend
3
6A
6C
8
12F
20
19A
PMEN Clone

Figure 2A: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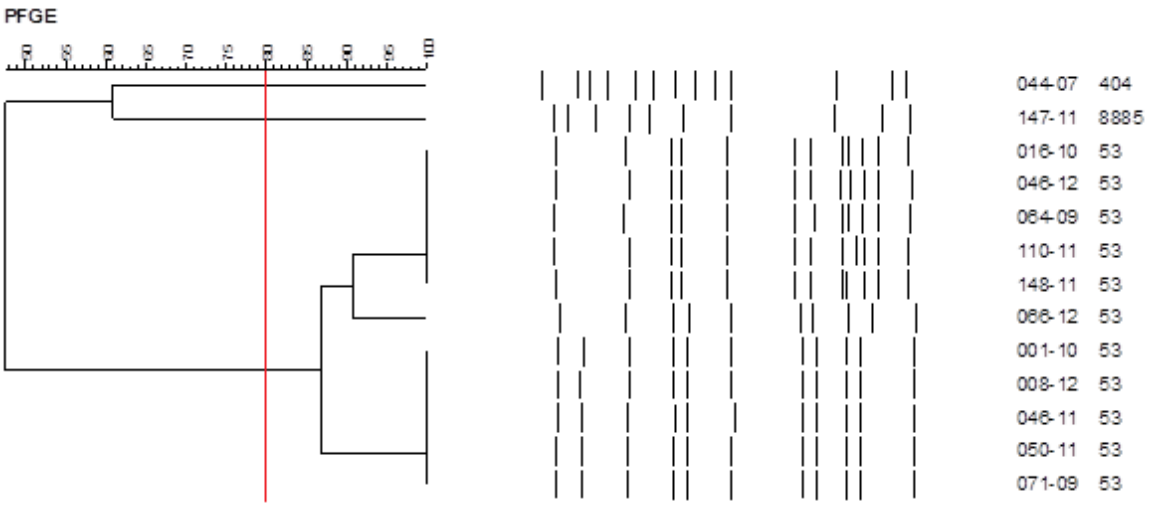
041-09 180
118-11 180
025-10 180
040-11 180
122-11 180
163-11 180
133-12 180
020-12 180
025-09 180
003-12 180
111-11 180
036-09 180
061-11 180
012-11 505
032-07 505
021-12 505
003-09 1116
153-11 1116
029-12 180
006-10 72

Figure 2B:

Figure 2C:

Figure 2D: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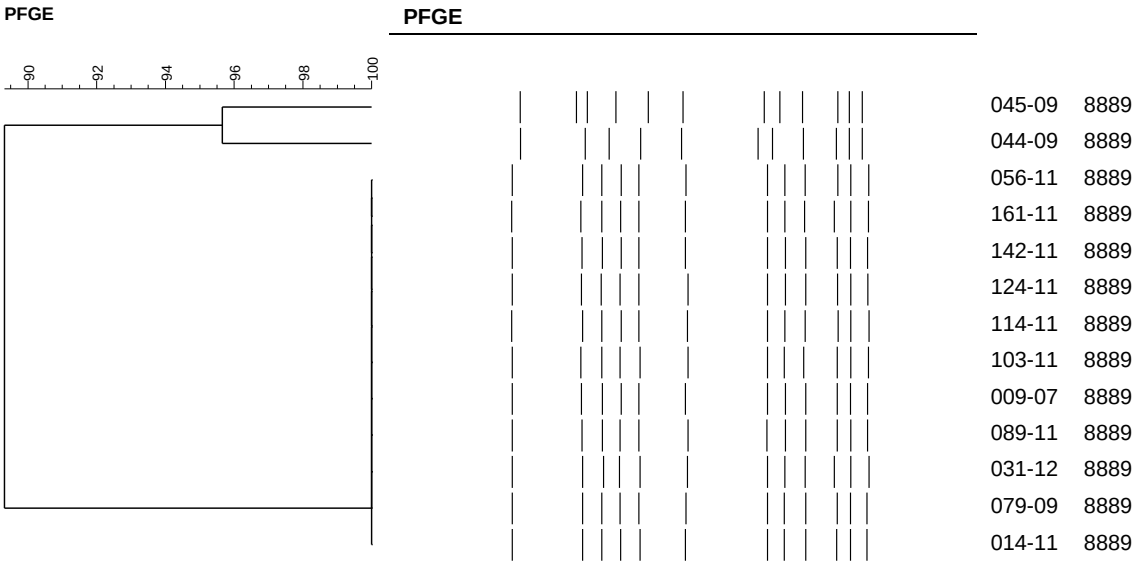
147-11 8885
016-10 53
046-12 53
064-09 53
110-11 53
148-11 53
066-12 53
001-10 53
008-12 53
046-11 53
050-11 53
071-09 53

Figure 2E: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96
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PFGE
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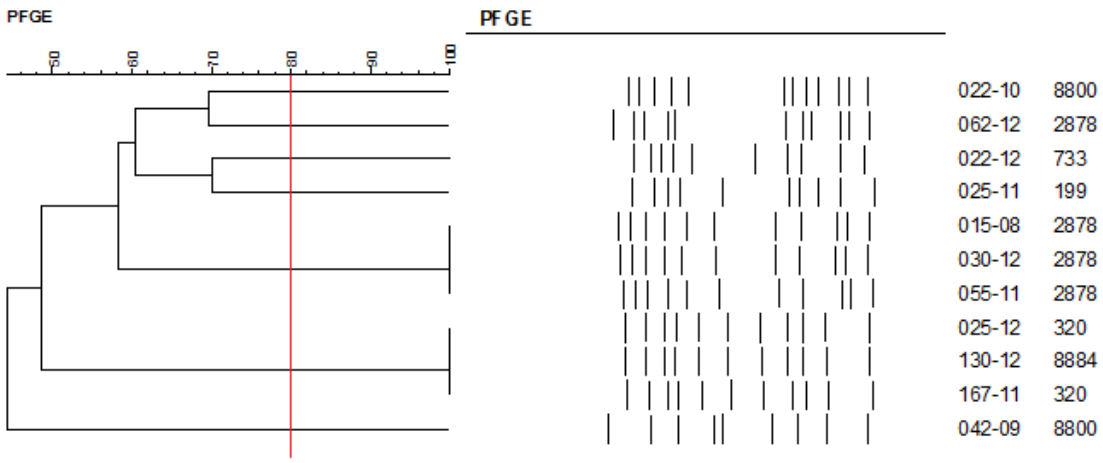
079-09 8889
014-11 8889

Figure 2F: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80
90
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PFGE
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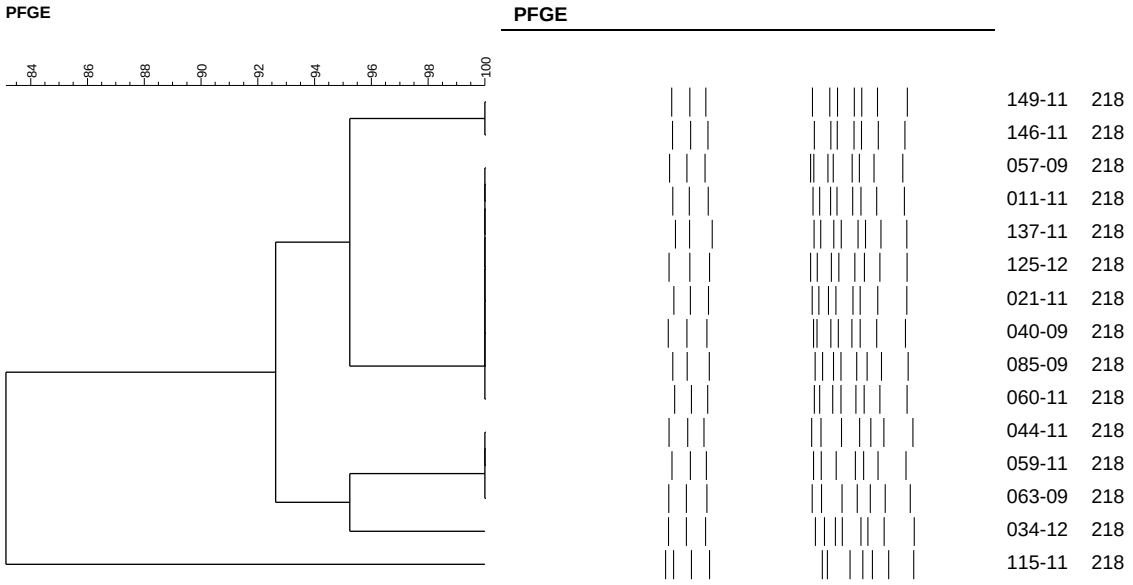
042-09 8800

Figure 2G: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057-09 218
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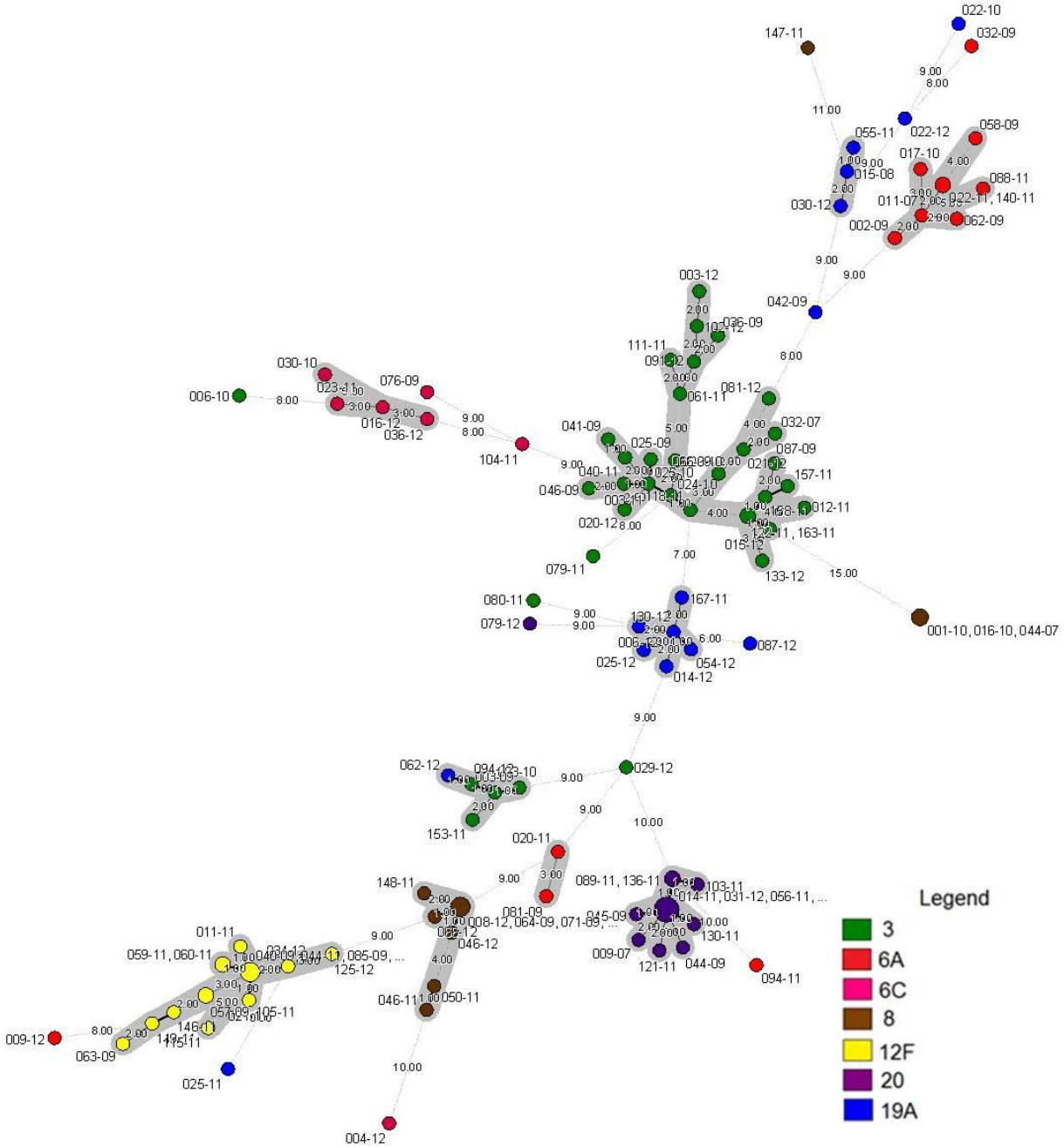
034-12 218
115-11 218

Figure 3:



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ANEXOS

4.1. Normas para publicação

JCM
Journals.ASM.org

August 2014, Instructions to Authors, pages 1–21

2014 INSTRUCTIONS TO AUTHORS

SCOPE

The *Journal of Clinical Microbiology* (JCM) is devoted to the dissemination of new knowledge concerning the laboratory diagnosis of human and animal infections. In addition, JCM is an appropriate forum for the publication of information related to the role of the laboratory in both the management of infectious diseases and the elucidation of the epidemiology of infections. Manuscripts which present the results of original scientific investigations are encouraged. The three principal attributes that we require of papers published in JCM are timeliness, relevance to the practice of clinical microbiology, and quality science. Manuscripts that present information that is largely only of relevance to a restricted geographic area are discouraged.

JCM welcomes submission of manuscripts that present the results of investigations pertaining to new technologies in clinical microbiology when they address new applications, substantially extend our understanding of the role of the technology in the clinical microbiology laboratory, or provide clinical or laboratory outcome data.

JCM will consider manuscripts which describe truly novel molecular methods for use in the diagnosis or elucidation of infection. However, we discourage submission of manuscripts predicated on the application of previously well-described methods (e.g., RT-PCR, RAPD analysis, PFGE, and real-time PCR, etc.), even though the application may be new. Papers employing established molecular methods will be considered only when the application is examined in comparison to some other existing diagnostic method(s). Such comparative studies should include information regarding assay sensitivity, specificity, and diagnostic accuracy.

Studies with matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) as a primary focus must offer something that is truly novel in order to merit consideration for publication in JCM, e.g., application of MALDI-TOF MS to previously unstudied organisms or organism groups, applications that extend beyond organism identification, clinical outcome studies, comparisons of different MALDI-TOF instruments, broad comparisons with conventional methods, or descriptions of modifications of existing MALDI-TOF MS methods. JCM will not consider manuscripts that describe routine applications of MALDI-TOF MS.

Papers submitted to JCM with whole-genome sequencing or the microbiome as a focus will be considered only when the studies performed are scientifically sound, the observations timely and novel, and the information presented of relevance to the practice of clinical microbiology. Similarly, papers that convey the results of meta-analysis studies will be considered when the data presented are extensive and when the observations made in the study are broadly applicable to the practice of clinical microbiology.

Case Reports will be considered if they are novel, add to existing knowledge, and are consistent with the primary objectives of the *Journal* as outlined above. (See "Case Reports" in the Organization and Format section and the "Case Report" paragraph under "Full-Length Papers" for descriptions of the two different types of Case Reports published.)

To ensure the completeness of investigations in which the performance of various diagnostic assays or methods is presented, authors are encouraged to refer to the Standards for the Reporting of Diagnostic Accuracy (STARD) for guidance. The entire set of guidelines, including checklists, may be found at http://stard-statement.org/.

ASM publishes a number of different journals covering various aspects of the field of microbiology. Each journal has a prescribed scope which must be considered in determining the most appropriate journal for each manuscript.

(i) With respect to antimicrobial agents, JCM will consider clinically relevant manuscripts (a) that pertain to *in vitro* susceptibility test methods; (b) that are concerned with quality control procedures related to antimicrobial susceptibility tests; (c) that deal with investigations of test methods aimed at measuring levels of antimicrobial agents in clinical specimens; or (d) that describe the use of antimicrobial agents as tools in the isolation, identification, or epidemiologic assessment of microorganisms associated with disease. Manuscripts pertaining to other aspects of antimicrobial agents, such as their basic mechanisms of action, the elucidation of resistance determinants, pharmacokinetics and pharmacodynamics, and the development of new agents, will be considered for publication in *Antimicrobial Agents and Chemotherapy*.

(ii) Manuscripts that present the results of investigations with a primary focus on the basic mechanisms of pathogenesis of microorganisms or the pathophysiology of infections should be directed to *Infection and Immunity* (for bacteria, parasites, and fungi) or the *Journal of Virology* (for viruses).

(iii) Reports of clinical microbiology investigations or studies of the hospital population and the environment as they relate to nosocomial infections should be submitted to JCM. Manuscripts dealing with ecology or environmental studies or with the application of microorganisms to agricultural or industrial processes are more appropriate for *Applied and Environmental Microbiology*.

(iv) Papers involving immunologic assays for use in the diagnosis or elucidation of infection, vaccines, or the assessment and laboratory diagnosis of immunologic diseases (e.g., autoimmune diseases and primary immunodeficiencies) should be submitted to *Clinical and Vaccine Immunology* (formerly *Clinical and Diagnostic Laboratory Immunology*).

Questions about these guidelines may be directed to the editor in chief of the journal being considered.

If transfer to another ASM journal is recommended by an editor, the corresponding author will be contacted.

Instructions to Authors are updated throughout the year. The current version is available at http://journalitas.asm.org/t/49544.

Note that a manuscript rejected by one ASM journal on scientific grounds or on the basis of its general suitability for publication is considered rejected by all other ASM journals.

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The Council Policy Committee (CPC) of the American Society for Microbiology affirms the long-standing position of the Society that microbiologists will work for the proper and beneficent application of science and will call to the attention of the public or the appropriate authorities misuses of microbiology or of information derived from microbiology. ASM members are obligated to discourage any use of microbiology contrary to the welfare of humankind, including the use of microbes as biological weapons. Bioterrorism violates the fundamental principles expressed in the Code of Ethics of the Society and is abhorrent to ASM and its members.

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Ethical Guidelines

ASM requirements for submitted manuscripts are consistent with the Recommendations for the Conduct, Reporting, Editing, and Publication of Scholarly Work in Medical Journals, as last updated by the International Committee of Medical Journal Editors in August 2013 (http://www.icmje.org/).

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Patient Identification

To protect the privacy of individuals mentioned in clinical studies, in case histories, and as sources of isolates, do not identify them by their initials, even as part of a strain designation. Change the initials to numerals or use randomly chosen letters. Do not give hospital unit numbers; if a designation is needed, use only the last two digits of the unit. (Note: established designations of some viruses and cell lines, although

they consist of initials, are acceptable [e.g., JC virus, BK virus, and HeLa cells].)

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Authors of manuscripts which contain quantitative real-time PCR applications are encouraged to consult the article concerning minimum information for publication of quantitative real-time PCR experiments (the MIQE guidelines) by Bustin et al. (Clin. Chem. **55:**611–622, 2009) for guidance as to what information should be considered for inclusion in their submission.

Newly determined nucleotide and/or amino acid sequence data must be deposited and GenBank/ENA/DDBJ accession numbers must be included in the manuscript no later than the modification stage of the review process. It is expected that the sequence data will be released to the public no later than the publication (online posting) date of the accepted manuscript. The accession numbers should be included in a separate paragraph at the end of the Materials and Methods section for full-length papers or at the end of the text for Short-Form papers. If conclusions in a manuscript are based on the analysis of sequences and a GenBank/ENA/DDBJ accession number is not provided at the time of the review, authors should provide the annotated sequence data as supplemental material not for publication.

It is expected that when previously published sequence accession numbers are cited in a manuscript, the original citations (e.g., journal articles) will be included in the References section when possible or reasonable.

Authors are also expected to do elementary searches and comparisons of nucleotide and amino acid sequences against the sequences in standard databases (e.g., GenBank) immediately before manuscripts are submitted and again at the proof stage.

Analyses should specify the database, and the date of each analysis should be indicated as, e.g., January 2014. If relevant, the version of the software used should be specified.

See "Presentation of Nucleic Acid Sequences" for nucleic acid sequence formatting instructions.

The URLs of the databases mentioned above are as follows: DNA Data Bank of Japan (DDBJ), http://www.ddbj.nig.ac.jp/; European Nucleotide Archive (ENA), http://www.ebi.ac.uk/ena/; and GenBank, National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/nucleotide.

Proper Use of Locus Tags as Systematic Identifiers for Genes

To comply with recommendations from the International Nucleotide Sequence Database (INSD) Collaborators and to avoid conflicts in gene identification, researchers should implement the following two fundamental guidelines as standards for utilization of locus tags in genome analysis, annotation, submission, reporting, and publication. (i) Locus tag prefixes are systematic gene identifiers for all of the replicons of a genome and as such should be associated with a single genome project submission. (ii) New genome projects must be registered with the INSD, and new locus tag prefixes must be assigned in cooperation with the INSD to ensure that they conform to the agreed-upon criteria.

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1. **Caserta E, Haemig HAH, Manias DA, Tomsic J, Grundy FJ, Henkin TM, Dunny GM.** 2012. *In vivo* and *in vitro* analyses of regulation of the pheromone-responsive *prgQ* promoter by the PrgX pheromone receptor protein. J. Bacteriol. **194:**3386–3394.
2. **Falagas ME, Kasiakou SK.** 2006. Use of international units when dosing colistin will help decrease confusion related to various formulations of the drug around the world. Antimicrob. Agents Chemother. **50:**2274–2275. (Letter.) {*"Letter" or "Letter to the editor" is allowed but not required at the end of such an entry.*}
3. **Cox CS, Brown BR, Smith JC.** J. Gen. Genet., in press.* {*Article title is optional; journal title is mandatory.*}
4. **da Costa MS, Nobre MF, Rainey FA.** 2001. Genus I. Thermus Brock and Freeze 1969, 295,[AL] emend. Nobre, Trüper and da Costa 1996b, 605, p 404–414. *In* Boone DR, Castenholz RW, Garrity GM (ed), Bergey's manual of systematic bacteriology, 2nd ed, vol 1. Springer, New York, NY.
5. **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.*}
6. **Forman MS, Valsamakis A.** 2011. Specimen collection, transport, and processing: virology, p 1276–1288. *In* Versalovic J, Carroll KC, Jorgensen JH, Funke G, Landry ML, Warnock DW (ed), Manual of clinical microbiology, 10th ed, vol 2. ASM Press, Washington, DC.
7. **Fitzgerald G, Shaw D.** *In* Waters AE (ed), Clinical microbiology, in press. EFH Publishing Co, Boston, MA.* {*Chapter title is optional.*}
8. **García CO, Paira S, Burgos R, Molina J, Molina JF, Calvo C, Vega L, Jara LJ, García-Kutzbach A, Cuellar ML, Espinoza LR.** 1996. Detection of *Salmonella* DNA in synovial membrane and synovial fluid from Latin American patients using the polymerase chain reaction. Arthritis Rheum. **39**(Suppl 9)**:**S185. {*Meeting abstract published in journal supplement.*}
9. **Carlson E.** 2013. Selective penicillin-binding protein imaging probes reveal substructure in bacterial cell division, p 59. Final Program 113th Gen. Meet. Am. Soc. Microbiol. American Society for Microbiology, Washington, DC. {*Abstract title is optional.*}
10. **Rotimi VO, Salako NO, Mohaddas EM, Philip LP.** 2005. Abstr. 45th Intersci. Conf. Antimicrob. Agents Chemother., abstr D-1658. {*Abstract title is optional.*}
11. **Green PN, Hood D, Dow CS.** 1984. Taxonomic status of some methylotrophic bacteria, p 251–254. *In* Crawford RL, Hanson RS (ed), Microbial growth on C_1 compounds. Proceedings of the 4th International Symposium. American Society for Microbiology, Washington, DC.
12. **O'Malley DR.** 1998. Ph.D. thesis. University of California, Los Angeles, CA. {*Title is optional.*}
13. **Odell JC.** April 1970. Process for batch culturing. US patent 484,363,770. {*Include the name of the patented item/process if possible; the patent number is mandatory.*}
14. **Elder BL, Sharp SE.** 2003. Cumitech 39, Competency assessment in the clinical laboratory. Coordinating ed, Sharp SE. ASM Press, Washington, DC.

*A reference to an in-press ASM publication should state the control number (e.g., JCM00123-14) if it is a journal article or the name of the publication if it is a book.

Online-only references must provide essentially the same information that print references do. For online journal articles, posting or revision dates may replace the year of publication; a DOI (preferred) or URL is required for articles with nontraditional page numbers or electronic article identifiers.

1. **Bina XR, Taylor DL, Vikram A, Ante VM, Bina JE.** 2013. *Vibrio cholerae* ToxR downregulates virulence factor production in response to cyclo(Phe-Pro). mBio **4**(5)**:**e00366-13. doi:10.1128/mBio.00366-13.
2. **Winnick S, Lucas DO, Hartman AL, Toll D.** 2005. How do you improve compliance? Pediatrics **115:**e718–e724. doi:10.1542/peds.2004-1133.
3. **Dionne MS, Schneider DS.** 2002. Screening the fruitfly immune system. Genome Biol. **3:**reviews1010-reviews1010.2. doi:10.1186/gb-2002-3-4-reviews1010.
4. **Giegé R, Springer M.** 2012. Aminoacyl-tRNA synthetases in the bacterial world. EcoSal Plus doi:10.1128/ecosalplus.4.2.1.

Note: a posting or accession date is required for any online reference that is periodically updated or changed.

Citations of ASM Accepts manuscripts should look like the following example.

Wang GG, Pasillas MP, Kamps MP. 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.

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 FX, Merianos HJ, Brunger AT, Engelman DM. 13 February 2001. Polar residues drive association of polyleucine transmembrane helices. Proc. Natl. Acad. Sci. U. S. A. doi:10.1073/pnas.041593698.

(ii) References cited in the text. References that should be cited in the text include

- 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

These references 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 and 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.state.micro.edu]).

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) Citations in abstracts. Because the abstract must be able to stand apart from the article, references cited in it should be clear without recourse to the References section. Use an abbreviated form of citation, omitting the article title, as follows.

(P. S. Satheshkumar, A. S. Weisberg, and B. Moss, J. Virol. 87:10700–10709, 2013, doi:10.1128/JVI.01258-13)
(J. H. Coggin, Jr., p. 93–114, *in* D. O. Fleming and D. L. Hunt, ed., *Biological Safety. Principles and Practices*, 4th ed., 2006)
". . . in a recent report by D. A. Hopwood [mBio 4(5): e00612-13, 2013, doi:10.1128/mBio00612-13] . . . ."

This style should also be used for Addenda in Proof.

(iv) References related to supplemental material. If references must be cited in the supplemental material, list them in a **separate** References section within the supplemental material and cite them by those numbers; do not simply include citations of numbers from the reference list of the associated article. If the same reference(s) is to be cited in both the article itself and the supplemental material, then that reference would be listed in both References sections.

Short-Form Papers

The Short-Form format is intended for the presentation of brief observations that do not warrant full-length papers. However, Short-Form papers should contain firm data; observations alone are not acceptable. Submit Short-Form papers 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.

The title, running title (not to exceed 54 characters and spaces), byline, and correspondent footnote should be prepared as for a full-length paper. Each Short-Form paper must have an abstract of no more than 50 words. Do not use section headings in the body of the Short Form; 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. Present acknowledgments as in full-length papers. 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 following invitation by the editor in chief of JCM. Unsolicited Minireviews are discouraged. A topical outline should be provided to the editor in chief for approval prior to submission of the completed Minireview manuscript in the eJP online manuscript submission and peer review system.

Minireviews are not expected to be comprehensive reviews of the literature but rather focused discussions of specific topics. A standard title page should be provided. This is followed by an abstract of 100 words or less and then the text of the Minireview, which should not exceed 12 double-spaced manuscript pages in length, exclusive of tables, figures, photographs, and references. Up to three tables, figures, or photographs, total, may be included. References should be limited to no more than 30. 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 and online ahead of print 6 to 8 weeks earlier.

Author bio. A short biographical sketch and photograph of the **one** author most responsible for the minireview should be submitted along with the initial version of the manuscript. These will be published at the end of the article.

- The text limit is 150 words and should include WHO you are (your name), WHERE you received your education, WHAT positions you have held and at WHICH institutions, WHERE you are now (your current institution), WHY you have this interest, and HOW LONG you have been in this area, as well as a brief review of your scholarly interests and record of publication. In addition, please list pertinent significant awards you have received.
- The photo should be a recent black-and-white head shot of passport size. It will be reduced to approximately 1.125 inches wide by 1.375 inches high. The photo must meet the production criteria for regular figures and should be checked for production quality by using Rapid Inspector, provided at the following URL: http://rapidinspector.cadmus.com/RapidInspector/zmw/index.jsp.
- To submit, upload the text and photo with your manuscript in the submission and review system. Include the biographical text immediately **after** the References section of your manuscript, in the same file. It should be labeled with the heading "Biosketch." Upload the head shot photograph in the submission system as a "Minireview Bio Photo"; **include the author's name or enough of it for identification in the photo's file name.**

Contact the scientific editor if you have questions about what to write. Contact the production editor if you have questions about submitting your files.

Commentaries

Commentaries are invited communications concerning topics relevant to the readership of JCM and are intended to engender discussion. Reviews of the literature, methods and other how-to papers, and responses targeted at a specific published paper are not appropriate. Commentaries are subject to review.

The length may not exceed four printed pages, and the format is like that of a Minireview (see above) except that the abstract is limited to 75 words.

Point-Counterpoint

Point-Counterpoint is a feature of JCM in which two experts present opposing views on a contemporary issue in the laboratory diagnosis of infectious diseases. This feature will be the lead article in the issue of JCM in which it appears. Participation as an author of a Point-Counterpoint feature is by invitation only.

A JCM editor will write a brief introductory piece of approximately 200 words outlining why a specific issue is important and then present the issue in the form of a question. The two experts will then each write a commentary, no more than 1,000 words in length, in which they present evidence in support of either the pro or con view. One table or one figure may be included. Since these discussions will be evidence based, authors may also cite up to 10 references. Unpublished or in-press data which reflect the current practice in their laboratory may be used but should not be the sole basis for their position.

Authors should send commentaries directly back to the JCM editor within 30 days of receipt of the introductory statement. Following receipt of both the pro and con commentaries, the editor will review the submissions and may return them to the author(s) with comments and/or suggested revisions. If revisions are required, the author(s) will have 14 days to craft a revised commentary, which will be sent directly back to the editor. Upon receipt of final commentaries, the JCM editor will write a brief summary consisting of no more than six one-sentence bullet points, outlining where the experts agree (no more than three points) and disagree (no more than three points). The JCM editor will then upload the introduction, both commentaries, and the summary in eJP.

Case Reports

While a full-length article or a Short-Form paper 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.

Photo Quiz

A Photo Quiz submission should present the findings of some relevant, interesting, and new observation pertinent to the practice of clinical microbiology in which a photograph is particularly useful in conveying important information **and** where the observation can serve as the basis for both a question and an answer. The photograph may be of a micrograph, some other laboratory material, a clinical lesion, or the results of an imaging study.

A Photo Quiz consists of two parts: (i) a case presentation featuring a photograph depicting some unusual and/or informative finding in clinical microbiology and (ii) an answer to the quiz. The case presentation and the answer must be submitted as two separate articles. Note that authors and affiliations are listed below the title.

Photo Quiz case presentation. The text in the Photo Quiz case presentation should be limited to 200 to 300 words. The header for the case presentation should read "Photo Quiz."

Please include a photograph about 39 picas (6.5 inches) wide and 28 picas (4.625 inches) high. Since photos appearing with published Photo Quizzes appear on the cover of the journal, a high-resolution TIFF or EPS file is preferred. A short legend for the photo must be provided, and the photo must be cited in the case presentation. Refer to a recently published Photo Quiz for correct formatting.

Answer to Photo Quiz. The text of the answer to the Photo Quiz should also be limited to 200 to 300 words. The header to the answer should read "Answer to Photo Quiz." Four to six references may be cited at the end of the Photo Quiz answer.

Submission. The Photo Quiz case presentation should be submitted in the "Photo Quiz" manuscript category. The Photo Quiz answer should be submitted in the "Photo Quiz Answer" manuscript category.

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 Short-Form papers.

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 below the title.

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/she 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 according 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 the eJP online manuscript submission and peer review system.

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 publication (e.g., a misspelling, a dropped word or line, or mislabeling in a figure) of a published article. Submit Errata via the eJP online manuscript submission and peer review system (see "Submission, Review, and Publication Processes"). In the Abstract section of the submission form (a required field), put "Not Applicable." Upload the text of your Erratum as a Microsoft Word file. Please see a recent issue for correct formatting.

Author Corrections

The Author 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 [noncritical] portion 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 Correction via the eJP online manuscript submission and peer review system (see "Submission, Review, and Publication Processes"). Select Author Correction as the manuscript type. In the Abstract section of the submission form (a required field), put "Not Applicable." Upload the text of your Author Correction as a Microsoft Word file. Please see a recent issue for correct formatting. Signed letters of agreement must be supplied as supplemental material not for publication (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 the eJP online manuscript submission and peer review system (see "Submission, Review, and Publication Processes"). In the Abstract section of the submission form (a required field), put "Not Applicable." Upload the text of your Retraction as a Microsoft Word file. Letters of agreement signed by all of the authors must be supplied as supplemental material not for publication (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 chairperson of the ASM Journals 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

Illustrations

Image manipulation. Digital images submitted for publication may be inspected by ASM production specialists for any manipulations or electronic enhancements that may be considered to be the result of scientific misconduct based on the guidelines provided below. Any images/data found to contain manipulations of concern will be referred to the editor in chief, and authors may then be requested to provide their primary data for comparison with the submitted image file. Investigation of the concerns may delay publication and may result in revocation of acceptance and/or additional action by ASM.

Linear adjustments to contrast, brightness, and/or color are generally acceptable, as long as the measures taken are necessary to view elements that are already present in the data and the adjustments are applied to the entire image and not just specific areas. Unacceptable adjustments to images include, but are not limited to, the removal or deletion, concealment, duplication (copying and pasting), addition, selective enhancement, or repositioning of elements within the image.

Nonlinear adjustments made to images, such as changes to gamma settings, should be fully disclosed in the figure legends at the time of submission. In addition, images created by compiling multiple files, including noncontiguous portions of the same image, should clearly distinguish that these multiple files are not a single image. This can be done by "tooling," or inserting thin lines, between the individual images.

File types and formats. 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.

On initial submission, figures may be uploaded as individual PDF files or combined and uploaded as a single PDF file. Place each legend in the text file, as well as on the same page with the figure to assist review. At the modification stage, production-quality digital files must be provided. The legends will be copy-edited and typeset for final publication and should not be included as part of the figure itself at this stage. All graphics submitted with modified manuscripts must be bitmap, grayscale, or in the RGB (preferred) or CMYK color mode. See "Color illustrations." Halftone images (those with various densities or shades) must be grayscale, not bitmap. JCM accepts TIFF or EPS files but discourages PowerPoint for either black-and-white or color images.

For instructions on creating acceptable EPS and TIFF files, refer to the Cadmus digital art website, http://art.cadmus.com/da/index.jsp. 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. For proper font use in PowerPoint images, refer to the Cadmus digital art website, http://art.cadmus.com/da/instructions/ppt_disclaimer.jsp. Note that, due to page composition system requirements, you must verify that your PowerPoint files can be converted to PDF without any errors.

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, Web-based application that identifies file characteristics that may render the image unusable for production.

If you have additional questions about using the Rapid Inspector preflighting tool, please send an e-mail inquiry to helpdesk.digitalartsupport@cenveo.com.

Minimum resolution. It is extremely important that a high enough resolution is used. All separate images that you import into a figure file must be at the correct resolution before they are placed. (For instance, placing a 72-dpi image in a 300-dpi EPS file will not result in the placed image meeting the minimum requirements for file resolution.) 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 combination art (lettering and images)
- 1,200 dpi for line art

Size. All graphics **should 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: 20.6 picas (ca. 8.7 cm)
- Maximum width for a 2-column figure: 42 picas (ca. 17.8 cm)
- Minimum width for a 2-column figure: 26 picas (11.1 cm)
- Maximum height for a standard figure: 54.7 picas (ca. 23.2 cm)
- Maximum height for an oversized figure (no running title); 57.4 picas (ca. 24.3 cm)

Contrast. Illustrations must contain sufficient contrast to be viewed easily on a monitor or on the printed page.

Labeling and assembly. All final lettering and labeling must be incorporated into the figures. On initial submission, illustrations should be provided as PDF files, with the legends in the text file and with a legend beneath each image to assist review. At the modification stage, production-quality digital figure

files (without legends) must be provided. Put the figure number 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: Arial, Helvetica, Times Roman, European PI, Mathematical PI, or Symbol. Courier may be used but should be limited to nucleotide or amino acid sequences, where a nonproportional (monospace) font is required. All fonts other than these 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.

Color illustrations. Color costs must be borne by the author. See "Publication Fees." All figures submitted in color will be processed as color. Adherence to the following guidelines will help to minimize costs and to ensure color reproduction that is as accurate as possible.

The final online version is considered the version of record for JCM and all other ASM journals. To maximize online reproduction, color illustrations should be supplied in the RGB color mode as either (i) RGB TIFF images with a resolution of at least 300 pixels per inch (raster files, consisting of pixels) or (ii) Illustrator-compatible EPS files with RGB color elements (vector files, consisting of lines, fonts, fills, and images). CMYK files are also accepted. Other than in color space, CMYK files must meet the same production criteria as RGB files. The RGB color space is the native color space of computer monitors and of most of the equipment and software used to capture scientific data, and it can display a wider range of colors (especially bright fluorescent hues) than the CMYK (cyan, magenta, yellow, black) color space used by print devices that put ink (or toner) on paper. For the print version (and reprints), ASM's print provider will automatically create CMYK versions of color illustrations from the supplied RGB versions. Color in the print journal may not match that in the online journal of record because of the smaller range of colors capable of being reproduced by CMYK inks on a printing press. For additional information on RGB versus CMYK color, refer to the Cadmus digital art site, http://art.cadmus.com/da/guidelines_rgb.jsp.

Drawings

Submit graphs, charts, complicated chemical or mathematical formulas, diagrams, and other drawings as finished products not requiring additional artwork or typesetting. 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:

(i) **All art must be submitted at its intended publication size.** For acceptable dimensions, see "Size," above.

(ii) **Avoid using screens (i.e., shading) in line art.** It can be difficult and time-consuming to reproduce these images without moiré 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,

(a) Generate the image at line screens of 85 lines per inch or less.

(b) When applying multiple shades of gray, differentiate the gray levels by at least 20%.

(c) Never use levels of gray below 5% or above 95%, as they are likely to fade out or become totally black when output.

(iii) Use thick, solid lines that are no finer than 1 point in thickness.

(iv) No type should be smaller than 6 points at the final publication size.

(v) Avoid layering type directly over shaded or textured areas.

(vi) Avoid the use of reversed type (white lettering on a black background).

(vii) Avoid heavy letters, which tend to close up, and unusual symbols, which the printer may not be able to reproduce in the legend.

(viii) 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 Système International d'Unités (SI) symbols (μ for 10^{-6}, m for 10^{-3}, k for 10^{3}, and M for 10^{6}, etc.). Thus, a representation of 20,000 cpm on a figure ordinate should be made by the number 20 accompanied by the label kcpm. 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*, 3rd ed. (RSC Publishing, Cambridge, United Kingdom, 2011); an abbreviated list is available at http://old.iupac.org/reports/1993/homann/index.html.

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 should be "2" and the label should be "10^{4} cells per ml" (not "cells per ml $\times$ 10^{-4}"). Likewise, an enzyme activity of 0.06 U/ml might be shown as 6 accompanied by the label 10^{-2} U/ml. The preferred designation is 60 mU/ml (milliunits per milliliter).

Presentation of Nucleic Acid Sequences

Long 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 or 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, and 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

On initial submission, each legend should be placed in the text file *and* be incorporated into the image file beneath the figure to assist review.

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 Microsoft 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.

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 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.

TABLE 1 Distribution of protein and ATPase in fractions of dialyzed membranes[a]

Membrane	Fraction	ATPase	
		U/mg of protein	Total U
Control	Depleted membrane	0.036	2.3
	Concentrated supernatant	0.134	4.82
E1 treated	Depleted membrane	0.034	1.98
	Concentrated supernatant	0.11	4.6

[a] Specific activities of ATPase of nondepleted membranes from control and treated bacteria were 0.21 and 0.20, respectively.

NOMENCLATURE

Chemical and Biochemical Nomenclature

The recognized authority for the names of chemical compounds is *Chemical Abstracts* (CAS; http://www.cas.org/) and its indexes. *The Merck Index*, 15th ed. (RSC Books, Cambridge, UK, 2013), 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*.

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 its supplements and at http://www.chem.qmul.ac.uk/iubmb/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. Authors of papers describing enzymological studies should review the standards of the STRENDA Commission for information required for adequate description of experimental conditions and for reporting enzyme activity data (http://www.beilstein-institut.de/en/projekte/strenda/guidelines/).

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 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* Typhimurium, *S.* Typhimurium, or *Salmonella* serovar Typhimurium. For other information regarding serovar designations, see *Antigenic Formulae of the Salmonella Serovars*, 9th ed. (P. A. D. Grimont and F.-X. Weill, WHO Collaborating Centre for Reference and Research on Salmonella, Institut Pasteur, Paris, France, 2007; see http://www.pasteur.fr/ip/portal/action/WebdriveActionEvent/oid/01s-000036-089). 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., American Society for Microbiology, 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: Prokaryotic Nomenclature Up-to-Date (http://www.dsmz.de/bacterial-diversity/prokaryotic-nomenclature-up-to-date.html) and List of Prokaryotic Names with Standing in Nomenclature (http://www.bacterio.net/). 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*, 5th ed. (C. P. Kurtzman, J. W. Fell, and T. Boekhout, ed., Elsevier Science, Amsterdam, Netherlands, 2011), and *Dictionary of the Fungi*, 10th ed. (P. M. Kirk, P. F. Cannon, D. W. Minter, and J. A. Stalpers, ed., CABI International, Wallingford, Oxfordshire, United Kingdom, 2008); 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 reported on the ICTV Virus Taxonomy website (http://www.ictvonline.org/index.asp). 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, as with 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 or laboratory, etc., in the 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.

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 of the *Journal of Bacteriology*.

"Mutant" versus "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 have 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.

Locus tags. Locus tags are systematic, unique identifiers that are assigned to each gene in GenBank. All genes mentioned in a manuscript should be traceable to their sequences by the reader, and locus tags may be used for this purpose in manuscripts to identify uncharacterized genes. In addition, authors should check GenBank to make sure that they are using the correct, up-to-date format for locus tags (e.g., uppercase versus lowercase letters and the presence or absence of an underscore, etc.). Locus tag formats vary between different organisms and also may be updated for a given organism, so it is important to check GenBank at the time of manuscript preparation.

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. FlyBase (http://flybase.org/) is the genetic nomenclature authority for *Drosophila melanogaster*. WormBase (http://www.wormbase.org/#01-23-6) is the genetic nomenclature authority for *Caenorhabditis elegans*. When naming genes for *Aspergillus* species, the nomenclature guidelines posted at http://www.aspergillus.org.uk/indexhome.htm?secure/sequence_info/nomenclature.htm should be followed, and the *Aspergillus* Genome Database (http://www.aspgd.org/) should be searched to ensure that any new name is not already in use. The *Saccharomyces* Genome Database (http://www.yeastgenome.org/) and the *Candida* Genome Database http://www.candidagenome.org/) are authorities for *Saccharomyces cerevisiae* and *Candida albicans* genetic nomenclature, respectively. For more 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 shows 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 *How To Write and Publish a Scientific Paper*, 7th 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, and Leu, etc.) may also be used.

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 Système International d'Unités (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, and GTP, etc. (for the respective 5′ phosphates of adenosine and other nucleosides) (add 2′-, 3′-, or 5′- when needed for contrast)

ATPase and dGTPase, etc. (adenosine triphosphatase and 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) and poly(dT), etc. (polyadenylic acid and 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[β-aminoethyl ether]-*N*,*N*,*N*′,*N*′-tetraacetic acid)
HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)
PCR (polymerase chain reaction)
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)
approx (approximately)
avg (average)
concn (concentration)
diam (diameter)
expt (experiment)
exptl (experimental)
ht (height)
mo (month)
mol wt (molecular weight)
no. (number)
prepn (preparation)
SD (standard deviation)
SE (standard error)
SEM (standard error of the mean)
sp act (specific activity)
sp gr (specific gravity)
temp (temperature)
tr (trace)
vol (volume)
vs (versus)
wk (week)
wt (weight)
yr (year)

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

Antibacterial agents. Use the indicated abbreviations for the following 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)
vancomycin (VAN)

β-Lactamase inhibitors. Use the indicated abbreviations for the following β-lactamase inhibitors.

clavulanic acid (CLA)
sulbactam (SUL)
tazobactam (TZB)

Antifungal agents. Use the indicated abbreviations for the following antifungal agents.

amphotericin B (AMB)
clotrimazole (CLT)
flucytosine (5FC)
fluconazole (FLC)
itraconazole (ITC)
ketoconazole (KTC)
nystatin (NYT)
terbinafine (TRB)
voriconazole (VRC)

Antiviral agents. Use the indicated abbreviations for the following antiviral agents.

acyclovir (ACV)
cidofovir (CDV)
famciclovir (FCV)
foscarnet (FOS)
ganciclovir (GCV)
penciclovir (PCV)
valacyclovir (VCV)
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, μ, 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 mμ or μμ. Use μg/ml or μg/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 μg or 10 min. For example, "pmol/min" is preferable to "nmol/10 min," and "μmol/g" is preferable to "nmol/μg." It is also preferable that an unambiguous form, such as exponential notation, be used; for example, "μmol g^{-1} min^{-1}" is preferable to "μmol/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 articles by Olsen (Infect. Immun. **71:**6689–6692, 2003; Infect. Immun. **82:**916–920, 2014).

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.

(i) Statistical analyses were performed on all quantitative data regardless of how significant the differences look in the tables or figures.

(ii) Data were appropriately analyzed as parametric (normally distributed) or nonparametric data.

(iii) 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, and 10th and 90th percentiles, etc.) are appropriate for nonparametric statistics (Mann-Whitney test and Kruskal-Wallis test, etc.). Means have no meaning in nonparametric analyses.

(iv) 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.

(v) 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.

(vi) Data presented as endpoints (i.e., LD_{50} and 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.

(vii) 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 or growth curves, etc.), one cannot use analyses such as *t* tests, ANOVA, or 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 or survival analysis, etc.

(viii) Statistical significance and biological significance are not the same. There is nothing magical about a *P* value of 0.05. When results from large sample sizes are compared, a *P* value of <0.05 will often be obtained, as *P* value is a function of both sample size and effect size. If sample sizes are large, then more-rigorous (i.e., smaller) *P* values may be desirable. If sample sizes are small, *P* values of >0.05 may still 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. **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
[α-^{14}C]lysine	[γ-^{32}P]ATP

4.2. Parecer de Aprovação no CEPE

Parecer Consubstanciado de Projeto de Pesquisa

Título do Projeto: Caracterização Genotípica de Isolados de Streptococus Pneumoniae Pertencentes a Sorotipos Não Integrantes de Vacina 10 -Valente

Pesquisador Responsável Cícero Dias **Parecer 1843/12**

Data da Versão 06/07/2012 | **Cadastro 462/12** | **Data do Parecer 27/09/2012**

Grupo e Área Temática III - Projeto fora das áreas temáticas especiais

Objetivos do Projeto
Avaliar os genótipos de pneumococus dos principais sorotipos que não integram a vacina 10-valente em dois períodos de tempo: antes e após a utilização dos mesmos.

Sumário do Projeto
Sendo o objetivo avaliar os genótipos de pneumococus dos principais sorotipos que não integram a vacina 10-valente em dois períodos de tempo. Para isso, isolados pertencentes aos sorotipos não integrantes da vacina 10-valente, procedentes de pacientes com doença pneumocócica invasiva (DPI) internados no Hospital Nossa Senhora da Conceição entre 2009 e 2012 serão identificados, sorotipados e terão sua variabilidade genética comparada através das técnicas PFGE e MLVA, afim de caracterizar os clones presentes neste hospital antes e após a introdução fa vacina 10-valente.

Itens Metodológicos e Éticos	Situação
Título	Adequado
Autores	Comentário
Local de Origem na Instituição	Adequado
Projeto elaborado por patrocinador	Não informado
Aprovação no país de origem	Não necessita
Local de Realização	Própria instituição
Outras instituições envolvidas	Sim
Condições para realização	Adequadas

Comentários sobre os itens de Identificação

Dissertação de Mestrado de Gabriela Rosa da Cunha.
Co-orientador: Pedro Álvares de Azevedo.

Introdução	Adequada

Comentários sobre a Introdução

Objetivos	Adequados

Comentários sobre os Objetivos

Pacientes e Métodos	
Delineamento	Adequado
Tamanho de amostra	Total Local
Cálculo do tamanho da amostra	Adequado
Participantes pertencentes a grupos especiais	Não
Seleção eqüitativa dos indivíduos participantes	Não se aplica
Critérios de inclusão e exclusão	Adequados
Relação risco- benefício	Não se aplica
Uso de placebo	Não utiliza
Período de suspensão de uso de drogas (wash out)	Nâo utiliza
Monitoramento da segurança e dados	Adequado
Avaliação dos dados	Adequada - quantitativa
Privacidade e confidencialidade	Adequada
Termo de Consentimento	Adequado
Adequação às Normas e Diretrizes	Sim

Comentários sobre os itens de Pacientes e Métodos

Cronograma	**Adequado**
Data de início prevista	**jan 2012**
Data de término prevista	**dez 1013**
Orçamento	**Adequado**
Fonte de financiamento externa	**Agência de fomento**

Comentários sobre o Cronograma e o Orçamento

Contemplado no Edital MCT?CNPQ 014/2008 Universal Edital Fapergs/CNPq 008/2009. Programa de Apoio a Nùcleos de Excelência Pronex e Edital Pronem Fapergs/CNPq nº 0031 2011 Programa de Apoio a Núcleos Emergentes.

Referências Bibliográficas	**Adequadas**

Comentários sobre as Referências Bibliográficas

Recomendação

Aprovar

Comentários Gerais sobre o Projeto

Tendo sido atendida a solicitação deste Comitê em relação à segurança e transporte das amostras, o projeto foi aprovado.

4.3. Artigo publicado durante o mestrado relacionado ao tema da dissertação

Antimicrobial resistance in *Streptococcus pneumoniae*: mechanisms and current epidemiology

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ABSTRACT

Infections caused by *Streptococcus pneumoniae* are a worrisome public health problem worldwide. Young children and the elderly are the main age groups affected and the highest burden of the disease is found in developing countries. Pneumococcal infections cause 11% of the total infant deaths, representing the leading cause of child death currently preventable by vaccination. Epidemiologic information about pneumococci in Brazil is somehow restricted, but available data reinforce the worrisome occurrence of pneumococcal diseases, which are commonly treated empirically. Limitations in the diagnostic methods, along with the severity of disease contribute to this behavior. Thus, surveillance studies are crucial to define the prevalence of resistant strains both globally and in a particular region, as these strains may compromise empirical therapeutic choices. However, although different clones of penicillin non-susceptible pneumococci are internationally distributed, and considering diseases other than meningitis, the prevalence of resistance to penicillin is quite low, making this old, safe, and inexpensive drug an attractive first choice to treat pneumococcal infections. The widespread use of conjugate vaccines among children, influencing the circulation of resistant clones and the distribution of serotypes reinforces the need of surveillance studies to define the prevalence of resistance.

Keywords: *Streptococcus pneumoniae; public health; antimicrobial resistance*

Infections caused by *Streptococcus pneumoniae* are a public health problem worldwide, especially considering young children and the elderly, and developing countries are clearly the most affected regions. Pneumococcal disease presents with a variable degree of severity, ranging from mild infections (acute otitis, sinusitis, and uncomplicated pneumonia) to invasive pneumococcal disease (IPD), such as bacteremic pneumonia and meningitis, which are associated with elevated morbidity and mortality, even when treated adequately. Indeed, case-fatality rates of pneumococcal meningitis can be as high as 37%[1] and around 20% of survivors experience long-term disabling sequelae[2].

Around 14.5 million episodes of severe pneumococcal disease occur annually in the world, causing 1,612,000 deaths[3], 825,000 of them among children under 5 years old, representing 11% of the total number of infant deaths[4]. Indeed, pneumococcal infections are the leading cause of child death currently preventable by vaccination[3].

Epidemiologic information about pneumococcal disease is lacking in many parts of Latin America. Information is mostly based on laboratory

surveillance of *S. pneumoniae* isolates from hospitalized patients with IPD, such as Pan-American Health Organization's SIREVA II database. It is estimated that around 20,200 to 33,000 children die annually in Latin America due to infections caused by pneumococci[3]. Brazilian data for pneumococcal disease are also scarce (considering the whole geographical area), but some studies from specific regions (Goiania, a Brazilian Midwest city) report that among children from 28 days to less than 3 years old, the incidence of IPD was 57.5/100,000 inhabitants from 2007 to 2009[5].

Pneumococci are the major cause of community-acquired pneumonia (CAP) and Brazil is among the 15 countries with the highest estimated numbers of new cases of pneumonia worldwide[6]. The mean rate of hospitalization due to CAP was 2,100/100,000 inhabitants in Brazil, from 2000 to 2008; 45% of them occurred in children aged less than 5 years and were caused by pneumococci[7].

According to the Brazilian Ministry of Health, it was notified of an average of 1,227 cases/year of pneumococcal meningitis, from 2000 to 2010, with a mortality rate around 30%[7]. Among children under 5 years of age, the incidence was 5.9/100,000 inhabitants, and this value increased to 9.5 cases per 100,000 inhabitants when younger children were taken into consideration (≤2 years old); mortality was high for both groups: 33 and 34%, respectively[5].

As an exclusively human pathogen, pneumococci colonize the nasopharynx, especially in children aged younger than 5, and transmission occurs by contact with respiratory secretions. From the primary colonization niche, they can migrate to other sites, such as middle ear, sinus, lung, blood, or cerebrospinal fluid and cause damage, leading to invasive disease. In this context, pneumococci have a robust arsenal of virulence factors, among which the polysaccharide capsule plays a central role[8]. Opsonophagocytosis is avoided by the presence of the capsule and differences in the polysaccharide composition distinguish over 94 distinct serotypes among pneumococci[9].

Commonly, antimicrobial therapy against pneumococci is defined empirically and the severity of the disease determines the medical approach[10]. Besides, specific features of microbiological diagnostic tests may also justify the empirical therapy. Indeed, some culture-based methods to identify *S. pneumoniae* have an intrinsic low sensitivity. For instance, blood cultures from patients with pneumococcal bacteremia detect the microorganism only in around 10 to 30% of cases[11]. On the other hand, some specimens, such as sputum, should be carefully analyzed, as low specificity may lead to false-positive results[11]. Another feature that substantiates the broad use of empirical therapy is the originally excellent activity of antimicrobials against pneumococci, especially the β-lactams. Pneumococci, in general, present very low Minimal Inhibitory Concentration (MIC) values[13] to these drugs.

However, this scenario has changed in recent years. The widespread and/or inadequate use of antimicrobials exerts a selective pressure on pneumococcal populations by picking out resistant isolates. In addition, selection of non-susceptible pneumococci may be a result of the dissemination of specific clones, which have an advantageous genetic background, including virulence and spread features, as well as resistance genes. The increased occurrence of these particular clones may be a natural event, where variations of frequency are expected during a certain period, or may be a result of another selective pressure force, such as vaccination[14].

Therefore, treatment of pneumococcal infections may be severely hampered by the isolation of non-susceptible strains. Indeed, it is well established that the delay in the implementation of the correct therapy in cases of CAP significantly increases in-hospital mortality, as well as 30-day mortality. Thus, it is reasonable to conclude pneumococcal resistance may directly affect patient's outcome[10].

RESISTANCE MECHANISMS

According to different guidelines, therapy against pneumococcal infections is primarily based on the use of β-lactams and macrolides[15-17]. Glycopeptides may also be an important therapeutic choice and their resistance among pneumococci has not been described so far. Some other drugs, such as fluoroquinolones, tetracycline, sulfamethoxazole-trimethoprim, lincosamines, and chloramphenicol also have good activity against pneumococci, but resistance against these drugs may occur in variable frequencies around the world as demonstrated bellow.

Defining resistance to some β-lactams (penicillins and cephalosporins) is a complex issue. The Clinical and Laboratory Standards Institute[18] determines breakpoints for the interpretation of antimicrobial susceptibility testing based on the

site of the infection (meningitis and non-meningitis) and the route of administration (oral and parenteral). The decision of the Institute to change breakpoints was based on the pharmacokinetic and pharmacodynamic properties of penicillin (low penetration across the blood-brain barrier). The term "penicillin non-susceptible *S. pneumoniae*" (PNSP) refers to isolates classified as resistant or intermediately resistant, according to current interpretative breakpoints[19]. Thus, according to CLSI meningitis criteria, pneumococci with MIC > 0.06 µg/mL are considered resistant to penicillin. On the other hand, by non-meningitis breakpoints, MICs of 4 µg/mL and ≥ 8 µg/mL define the isolate as intermediate and fully resistant to penicillin, respectively[18].

β-lactams act by binding to the penicillin-binding proteins (PBP), compromising cell wall formation, which leads to osmotic induced pneumococcal lysis[20]. This mechanism is highly effective and penicillin MICs for pneumococci are, in general, very low (as low as 0.01 µg/mL for benzylpenicillin). The first reports of higher penicillin MICs are from the 1960s and 1970s, but were neglected because of β-lactams excellent activity against pneumococci. Indeed, little regard was given to antimicrobial resistance in pneumococci until 1977 when the attention of the medical community was drawn to reports of an IPD epidemic in South Africa caused by highly resistant *S. pneumoniae*[21]. Following this report, multidrug-resistant (resistance to three or more antimicrobial classes) pneumococci were reported with greater frequency worldwide[20,22,23].

There are six physiological PBPs in the pneumococcal cell: PBP1a, PBP1b, PBP2a, PBP2b, PBP2x, and PBP3. Resistance to β-lactams is basically related to mutations in three of those enzymes: mutations in PBP2x and PBP2b being strongly related to resistance; mutated PBP1a, in tandem with PBP2b and PBP2x increases penicillin MICs further; altered PBP2a also seems to be related to resistance, but to a lesser degree[13,24,25].

While PBP genes are highly conserved among pneumococci susceptible to penicillin, in most clinical isolates of PNSP, PBPs are codified by mosaic genes, which are continuous nucleotide sequences that differ from the non-mosaic allele by up to 20%,[13] strongly suggesting a non-pneumococcal origin of these genes. Indeed, interspecies gene transfer, followed by recombination events involving closely related commensal species, such as *Streptococcus mitis* and *Streptococcus oralis* seems to be the origin of the mosaic PBP resistant genes[13,20].

Some of those mosaic genes have become stable in specific clones. For instance, some PNSP clones (Spain^{23F}-1, Spain^{6B}-2 and Spain^{9V}-3) are internationally disseminated (see section "international clones" below) and present a highly conserved PBP2x among them; this suggests that the mutated gene may generate some evolutionary advantage. Indeed, apart from the wondrously transformation potential of pneumococci, dissemination of resistant clones are also important for the increase of penicillin non-susceptibility among pneumococci[13].

PNSP will only disseminate if a delicate balance occurs between antimicrobial selective pressure and the cost that resistance imposes on bacterial fitness. In this context, mutations in *pbp2b* carry an energy expenditure that affects fitness. However, *pbp2b* mutants that also carry *pbp1a* and *pbp2x* mutated genes not only compensate energetic costs, but also increase MICs, leading to evolutionary better adapted bacterial isolates[13].

Along with PBP changes, mutations in non-PBP genes also occur in PNSP and, depending on the selective antibiotic, distinct genes are affected[19]. A mutation in the GlcNAc deacetylase (*pdgA*) has been detected by genome sequencing in high-level resistant transformants obtained in four selection steps using chromosomal DNA of a high-level resistant *S. pneumoniae* strain. Moreover, deletion of the peptidoglycan O-acetyl transferase has also been shown to cause an extensive reduction of resistance in several PNSP strains[26].

The worldwide increase in penicillin resistance coincided with an increase in macrolide resistance. In many parts of the world, macrolide resistance now exceeds penicillin[13]. Macrolides act by inhibiting protein synthesis as a result of their binding to the 23S portion of the ribosomal RNA[27]. Resistance is due to two major mechanisms: (i) alteration of the target site of the antimicrobial by producing a bacterial methylase codified by *erm* genes; (ii) expression of *mef* genes that codify an active efflux pump. Another mechanism may be associated with macrolide resistance but its clinical relevance is very low: through mutations in the 23S rRNA gene or in L4 and L22 ribosomal proteins[28,29].

Indeed, the production of methylase is the major mechanism of macrolide resistance and it commonly confers the MLS_B phenotype (resistance to Macrolides, Lincosamines and B Streptogramins). Two major acquired genes are responsible for this

resistance: *erm*(B) and *erm*(TR). The *erm*(TR) gene is a subclass of *erm*(A) and has a very narrow distribution compared to *erm*(B). The bacterium *Streptococcus pyogenes* seems to be the origin of *erm*(TR) and nasopharynx co-colonization with pneumococci. which may have allowed interspecies dissemination. Among pneumococci, after its first isolation, only a few isolates carrying this gene have been reported[13,30].

The presence of *erm*(B) usually leads to elevated MICs (> 64µg/mL). This gene is carried on members of Tn*916* family of transposons (in pneumococci: Tn*3872*, Tn*6002*, Tn*6003* and Tn*1545*), which also carry *tet*(M), an important determinant of tetracycline resistance. Although *tet*(M) is not always expressed, tetracycline resistance is very common among macrolide resistant pneumococci[27,31].

Clonal dissemination seems to play a more relevant role for macrolide resistance than gene acquisition by single strains as a result of the selective pressure of antimicrobial usage. Indeed, 77.1% of *erm*(B) are located into Tn*916* transposons family, suggesting that the increased occurrence of macrolide resistant pneumococci is a result of the clonal dissemination of these transposons. Indeed, an expressive number of pneumococci presenting macrolide resistance with the MLS_B phenotype (more than 50%) belong to a few international pneumococcal clones: Sweden^{15A}-25, Spain^{23F}-1, Spain^{6B}-2, clone^{19F}-ST276, and clone^{19A}-ST276 (see section "international clones" below)[32].

Efflux pump, codified by *mef* genes, confer lower macrolide MICs than *erm*(B): 1 to 32µg/mL. In this case, M phenotype (only macrolide resistance) occurs and lincosamines and B Streptogramins may have activity. Among pneumococci, there are three related genes: the abundant *mef*(E) and *mef*(A), and a third gene, *mef*(I), with a very narrow distribution so far[13]. *Mef*(E) and *mef*(A) show 90% of genetic identity and present a distinct geographical distribution: the former is widely distributed in USA, Asia, and South Africa, while the latter is more commonly recovered from European countries, as well as South America[33-35].

The increase in β-lactam resistance spurred the development of fluoroquinolones active against Gram-positive pathogens. Fluoroquinolone resistance is increasing amongst pneumococci. These drugs act by binding to the DNA gyrase (formed by GyrA and GyrB subunits) and topoisomerase IV (formed by ParC and ParE subunits) thus disrupting DNA synthesis. The primary target varies according to microorganisms (Gram-positive or Gram-negative) and the fluoroquinolone drug: among pneumococci, ciprofloxacin and levofloxacin act primarily in ParC topoisomerase subunit, while moxifloxacin firstly binds to GyrA DNA gyrase subunit[36].

Resistance to fluoroquinolone occurs because of gradual accumulation of point mutations in the Quinolone Resistance Determinant Region (QRDR) of the GyrA and/or ParC. Mutations in *parC* QRDR guarantee resistance to ciprofloxacin, but not to the newer fluorquinolones. Indeed, QRDR *parC* mutations are the primary step in fluorquinolone resistance. They do not substantially increase MICs but enhance the risk of new mutations. On the other hand, isolates presenting QRDR regions of *gyrA* and *parC* mutated have elevated MICs (> 16µg/mL). Mutations in *gyrB* and *parE* are infrequent and seem to be unexpressive[37-40].

Although some studies demonstrate an heterogeneous genetic background, fluoroquinolone resistance appears to be strongly associated with a single mutation in ParC and GyrA: substitution of a phenylalanine in positions 79 and 81, respectivelly. Indeed, a multicentric study demonstrated that 51% of pneumococci resistant to fluorquinolone showed only those point mutations[41].

Besides alterations in *parC* and *gyrA* nucleotide composition, the overexpression of efflux pumps, such as PmrA or the ABC pumps PatA and PatB, may have a role in fluorquinolone resistance. Although MICs in those isolates are not as high as the *gyrA* and *parC* mutated ones, overexpression of efflux pumps seems to increase chances of occurrence of point mutations[13].

Recombination does not play a central role in the dissemination of fluorquinolone resistance. Indeed, pneumococcal QRDR has been shown to have low homology with viridans QRDR, a species more frequently related to this phenotype than pneumococci[40]. In fact, some studies demonstrated the occurrence of mosaic genes shared by viridans and pneumococci but this was not common among the pneumococcal population. One reason for this may be bacterial fitness, even though supportive data are lacking. Unlike macrolide resistance, clonal dissemination of fluorquinolone resistance does not have a major participation in the increase of this resistance and, again, bacterial fitness may justify this observation[40].

Less clinically significant phenotypes among pneumococci include resistance to tetracycline, sulfamethoxazole plus trimethoprim, and

chloramphenicol. Tetracycline acts through binding to the 30S ribosomal unit, preventing protein synthesis. Resistance to this antimicrobial may be due to the presence of Tet(M) and, occasionally, Tet(O) proteins, which prevent tetracycline binding by a methylation reaction onto the target site; or, less frequently, the occurrence of efflux pumps, Tet(K) and Tet(L), respectivelly[41,42]. The *tet*(M) gene is located in genetic mobile elements widely found and transmitted among many Gram-positive bacteria justifying the frequent occurrence of this phenotype in pneumococcal population. Efficacy and low cost are the main reasons for sulfamethoxazole-trimethoprim therapy against pneumococci, especially in developing countries, where reports of resistance are increasing. Prophylactic usage of this antimicrobial to prevent secondary infections in HIV positive patients may also explain the high rates of resistance observed. Sulfamethoxazole-trimethoprim acts on folic acid synthesis and mutations in genes (*folA* and *folP*) that codify the binding target of these drugs are responsible for resistance[43]. Finally, chloramphenicol resistance occurs through production of chloramphenicol acetyltransferases, codified by *cat* genes. The enzyme converts the antimicrobial into a non-functional molecule, preventing chloramphenicol binding to 50S ribosomal subunit[44].

Table 1 summarizes the main resistance mechanisms to antimicrobials in *S. pneumoniae*.

PREVALENCE OF RESISTANCE

Global

Although the incidence of IPD caused by PNSP, pneumococci resistant to erythromycin or multiresistant pneumococci had decreased significantly after the introduction of the 7-valent pneumococcal conjugate vaccine (PCV7), including serotypes in 2000, the increased isolation of resistant non-vaccine serotypes promoted a rise in the frequency of resistant pneumococci in some parts of the world[45]. Indeed, from 1998 to 2003, the proportion of PNSP decreased from 32% to 19.4%, followed by a post-vaccine increase to 30.1% in 2005. Some serotypes are consistently related to the decrease of the susceptibility, especially the so-called pediatric serotypes (6A, 6B, 9V, 14, 19A, 19F, and 23F), as well as some other non-vaccine serotypes: 19A and 35B[46,47].

A recent multicentric study encompassing 2,173 IPD-recovered pneumococci from patients of all ages and from all continents (2004-2009) showed that 33.3% of all isolates were non-susceptible to penicillin (MIC < 0.06 µg/mL). Resistance to erythromycin was quite lower (22.9%) and 16.2% of all *S. pneumoniae* were resistant to both penicillin and erythromycin. Isolates resistant to levofloxacin represented only 0.5% of the total. Some serotypes were significantly associated with PNSP: 19A, 6A, 19F, 14, 6B, 9V, 35B, 23A, and 15A. Similarly, serotypes 19A, 6A, 15A, 19F, 9V, 6B, and 14 had a statistically significant relationship with erythromycin resistance[23].

If the populations with the highest risk for pneumococcal infections are taken into consideration[21], resistance to penicillin considerably increased in all continents compared to the general (all ages) population[23]. Brandon and Dowzicky included in their study pneumococci recovered from clinically relevant sites of pediatric populations (0 to 18 years old), from 2004 to 2011. Globally, PNSP was 46.1% and levofloxacin remained very low 0.3%[22].

Geographic occurrence of resistance is not homogeneous and both selective pressure by antimicrobial use and circulation of some specific clones/serotypes are responsible for the differences in the prevalence of resistant pneumococci worldwide[14].

Hackel et al. demonstrated that, for patients of all ages, erythromycin resistance ranges from 15.3% to 28.8% among all continents, with the lowest frequency in Latin America and the highest among Asian countries. On the other hand, Africa presents the highest frequency of isolation of PNSP (64.3%), while only 18.6% of pneumococci

Table 1: Mechanisms of resistance against the most clinically relevant antimicrobials.

Antimicrobial	Resistance mechanism	Reference
Penicillin	Mutations in *pbp* genes = mosaic genes	12
Erythromycin	Expression of *erm* (methylation) and/or *mef* (efflux pumps) genes	27
Tetracycline	Expression of *tet* genes: methylation [*tet*(M) and *tet*(O)] and/or efflux pump [*tet*(K) and *tet*(L)]	41
Fluoroquinolones	Mutations in QRDR of *par*C and *gyr*A.	35
Chloramphenicol	Expression of *cat* gene (acetyltransferases)	43
Sulfamethoxazole-trimethoprim	Mutations in *folA* and *folP*	42

recovered from European countries show this phenotype[23].

Geographical variation of frequency among the pediatric population presents a similar pattern. The lowest frequency of isolation of PNSP (34.4%) occurred in Europe, with a frequency almost twofold higher than that observed among all ages (18.6%). Africa had, once again, the highest rates of PNSP, 85.7%. All continents but Africa (no resistance detected) had levofloxacin-resistant pneumococci in a low frequency of isolation: from 0.2 in Europe to 1.1% in the Middle East[22].

Although data from Europe commonly demonstrate low rates of PNSP, a large recent European study including more than 21,000 pneumococcal isolates showed that some countries may have frequencies of PNSP and resistance to macrolides as high as 42.2% and 38.1%, respectively. Once again, serotypes 14, 19A, and 19F were the most commonly involved[48].

Some other limited studies have shown quite different frequencies of resistance, especially if differentiated populations are taken into consideration. For instance, pneumococci recovered from nasopharynx of healthy children in China revealed 39.4% of PNSP during 2012 to 2013[49]. In the same study, all 175 pneumococcal isolates were resistant to erythromycin. Over again, serotype 19F (precisely Taiwan1^{9F}-14 clone) was significantly associated with β-lactam resistance[49].

Resistance to erythromycin was also extremely high among Japanese pneumococcal isolates, recovered from noninvasive or colonization sites during 2011: 96.8%, and serotypes 23F and 6B were the most commonly related to this phenotype. On the other hand, resistance to penicillin was very low[50].

Other studies focusing on pneumococcal from carriage demonstrate a similar scenario[51-55], although some specific regions may show higher frequencies. Indeed, frequency of PNSP was 78.6% among isolates from healthy Korean children[56].

Of note, despite the worrisome occurrence of PNSP considering CLSI meningitis breakpoints, isolates presenting non-susceptibility to penicillin following non-meningitis breakpoints (MIC ≥4μg/mL) are very low worldwide[48].

BRAZIL

According to SIREVA II, the prevalence of PNSP (MIC > 0.06 μg/mL) in Brazil was 25.7%, while 11.5% showed erythromycin resistance[57]. Some regional studies present quite similar data. Of note, all studies used CLSI meningitis breakpoints to define PNSP.

Andrade et al. evaluated pneumococci recovered from children with IPD previous to implementation of vaccination in Brazil (207-2009) and identified 13.3% as PNSP, all of them belonging to serotypes included in PCV7. No levofloxacin resistant isolates were found and 13.3% presented macrolide resistance[5].

On the other hand, Mott et al. firstly evaluated 159 invasive pneumococcal isolates recovered in post-vaccination period (2010 to 2012) in the country. An increase of PNSP was observed (21.4%) compared to the above-cited study and serotypes 14, 9V, 19F, 23F, and 19A were the most commonly related to this phenotype. Only one isolate, belonging to serotype 19A, had a MIC=4 mg/mL to penicillin (intermediate resistance, according to CLSI criteria for non-meningitis), and isolates showing MICs≥8 mg/mL were not found. Resistance to erythromycin was 12% and only one isolate was resistant to fluoroquinolone[35].

Among pneumococci recovered from patients with meningitis during 2000-2007, the frequency of PNSP was very similar (22.2%) but erythromycin resistance was considerably lower: 0.8%[58]. Similar results were found when pneumococcal from carriage were taken into consideration[59].

Resistance rates to tetracycline, chloramphenicol, and sulfamethoxazole-trimethoprim were found to be more heterogeneous in different Brazilian studies[5,35,57,58,60,61]. As an example, non-susceptibility to sulfamethoxazole-trimethoprim varied from 28.5%[56] to 80%[5], while the percentage of pneumococci non-susceptible to tetracycline seems to be more homogeneous (around 20-30%)[35,58].

Continuous surveillance of pneumococci focusing on antimicrobial susceptibility, as well as serotype distribution is of great concern in developing countries such as Brazil and should be performed systematically to better understand the impact of vaccination on resistance rates.

PNEUMOCOCCAL INTERNATIONAL CLONES AND THE INFLUENCE OF VACCINATION IN THE DISSEMINATION OF RESISTANCE

Although *S. pneumoniae* is a genetically diverse species capable of expressing over 94 different capsular types[9], only a limited number of these serotypes associated with a few pandemic

clones have been responsible for the increase of pneumococcal drug resistance worldwide[14]. The origin of these drug resistant clones is believed to be the nasopharynx of young children, from where they are transferred from person-to-person. These circumstances, combined with frequent antibiotic use, constitute ideal conditions for the selection, amplification, and transmission of drug-resistant clones[62].

Created in 1997, the Pneumococcal Molecular Epidemiology Network (PMEN - http://web1.sph.emory.edu/PMEN/) intended to develop a global surveillance of antibiotic-resistant *Streptococcus pneumonia*e clones. In order to standardize the nomenclature and classification of these clones, their names are composed by the location of the first isolation, the serotype (superscript), plus a number indicating the chronological order of nomination by PMEN. For example, the first PMEN clone was isolated in Spain and the strains were serotyped as 23F: Spain^{23F}-1. Forty-three important disease-causing clones have been identified[63]. Although resistant strains are the primary focus of surveillance, some susceptible clones with relevant importance in invasive disease worldwide are also considered by PMEN. Table 2 presents characteristics of PMEN clones resistant to penicillin considering meningitis breakpoints (MIC> 0.06 µg/mL).

To be included into the network, clonality must be determined based on Pulsed-Field Gel Electrophoresis (PFGE), Multilocus Sequence Typing (MLST), and Penicillin-Binding Protein (PBP) fingerprinting results. Despite the high discriminatory power of PFGE, this technique has low reproducibility and, consequently, data may not be homogeneous among geographically distinct laboratories. On the other hand, MLST generates unambiguous data, making it easy to compare strains from different regions. Indeed, it increases the understanding of the pneumococcal population dynamics and their patterns of dissemination worldwide.

Apart from the β-lactams, resistance to erythromycin and tetracycline are the most prevalent phenotypes among PMEN clones (19/43; 44.2%).

Table 2: Pneumococcal Molecular Epidemiology Network clones presenting resistance to penicillin (meningitis breakpoints: MIC > 0.06 µg/mL).

PMEN clone	ST	Serotype	Vaccine	Susceptibility profile [c] (MIC in µg/mL)						
				PEN	CTX	ERY	CLI	CHL	TET	SXT
Spain^{23F}-1	81	23F	all [b]	1	1.5	0.25	0.19	16	64	2
Spain^{6B}-2	90	6B	all	0.5	0.75	0.19	0.19	16	48	2
Spain^{9V}-3	156	9V	all	1.5	0.75	0.125	0.19	3	0.5	1.5
Tennessee^{23F}-4	37	23F	all	0.125	32	32	0.125	3	0.25	2
Spain^{14}-5	18	14	all	1.5	1	0.19	0.125	32	24	0.25
Hungary^{19A}-6	268	19A	PCV13	1	0.75	>256	>256	24	48	3
S.Africa^{19A}-7	75	19A	PCV13	0.19	0.094	0.25	0.19	4	1	4
S.Africa^{6B}-8	185	6B	all	0.19	0.125	0.19	0.19	2	0.5	1.5
CSR^{14}-10	20	14	all	8	1	>256	>256	32	48	0.25
CSR^{19A}-11	175	19A	PCV13	6	0.5	>256	>256	24	64	2
Finland^{6B}-12	238	6B	all	4	0.75	>256	>256	8	64	6
S.Africa^{19A}-13	41	19A	PCV13	1.5	0.5	>256	>256	24	48	4
Taiwan^{19F}-14	236	19F	all	2	0.75	16	0.25	16	48	2
Taiwan^{23F}-15	242	23F	all	0.75	0.75	>256	>256	4	48	0.25
Poland^{23F}-16	173	23F	all	8	4	>256	0.25	16	64	1.5
Maryland^{6B}-17	384	6B	all	1.5	1	24	0.19	3	0.75	2
Tennessee^{14}-18	67	14	all	4	12	>256	>256	3	32	2
N.Carolina^{6A}-23	376	6A	PCV13	1	0.75	4	0.19	3	0.5	1
Utah^{35B}-24	377	35B	NONE	1	0.75	0.125	0.125	3	0.38	0.38
Denmark^{14}-32	230	14	PCV7	1	0.75	0.125	0.125	3	64	1.5
Norway^{NT}-42	344	NT	NONE	0.094	0.125	4	0.064	2	32	0.38
USA^{NT}-43	448	NT	NONE	0.094	0.094	4	0.094	3	32	0.25

[a] ST: sequence type, according to MLST results
[b] All = PCV7, PCV10 and PCV13
[c] * PEN = penicillin; CTX = ceftriaxone; ERY = erythromycin; CLI = clindamycin; CHL = chloramphenicol; TET = tetracycline; SXT = sulfamethoxazole-trimethoprim

Considering CLSI meningitis criteria for penicillin and ceftriaxone, 20 (46.5%) and 16 (37.2%) clones exhibit nonsusceptibility: MIC ≥ 0.12µg/mL and ≥ 2µg/mL, respectively. If non-meningitis breakpoints are taken into consideration, clones CSR^{14}-10, CSR^{19A}-11, Finland^{6B}-12, Poland^{23F}-16, and Tennessee^{14}-18 are non-susceptible to penicillin (MICs ≥ 4µg/mL). Poland^{23F}-16 and Tennessee^{14}-18 are also fully resistant to ceftriaxone (MICs ≥ 4µg/mL). Besides, despite its penicillin susceptibility, Tennessee^{23F}-4 is characterized by a very high ceftriaxone MIC (32 µg/ml). In general, these β-lactams nonsusceptible clones are multiresistant and their occurrence worldwide may strongly compromise empirical therapy against IPD.

PCV7 was particularly designed against the most prevalent and/or resistant serotypes, i.e. 4, 6B, 14, 18C, 19F, and 23F, which are widely distributed around the world. Indeed, most PMEN clones (51.2%) encompass pneumococci from PCV7, especially serotypes 14, 6B and 23F (17/22; 72.3%), which is not surprising, since vaccine formulations were developed precisely against the serotypes most commonly related to IPD worldwide and/or those with worrisome resistance characteristics. PCV10 (PCV7 serotypes plus 1, 5 and 7F) and PCV13 (PCV10 serotypes plus 3, 6A and 19A) comprise 65.1% (28/43) and 81.4% (35/43) of the international pneumococcal clones, respectively. Of note, 18.6% of PMEN clones are composed of serotypes not included in any vaccine formulation currently available (including two non-typable clones: Norway^{NT}-42 and USA^{NT}-43) and they will be further discussed below.

Among all 43 PMEN clones, the most widely distributed seem to be Spain^{23F}-1, Spain^{6B}-2, and Spain^{9V}-3. Spain^{23F}-1 predominantly circulates as a vaccine serotype 23F, multilocus sequence type 81 (ST81). However, ST81 has also been associated with several other serotypes, including both vaccine and non-vaccine types[64,65]. After the first isolation, Spain^{23F}-1 disseminated worldwide[64,66-69]. Indeed, by the late 1990s, it was estimated that approximately 40% of the penicillin non-susceptible pneumococci circulating in the USA were members of this clone[70], corroborating the spread of penicillin resistance determinants among other pneumococcal clones. Besides penicillin, Spain^{23F}-1 is also commonly associated with fluoroquinolone resistance and some authors have suggested that genetic determinants for this resistance have been donated from Spain^{23F}-1 to numerous unrelated pneumococcal clones[71].

It has been well demonstrated by Wyres et al. that Spain^{23F}-1 and related clonal variants (all belonging to the same clonal complex - CC81), exhibit extraordinary genetic diversity, which largely results from hundreds of recombination events[72]. These features indicate rapid genomic evolution and presumably allow rapid response to selective pressures such as those imposed by vaccine and antibiotic usage[65].

Indeed, although antibiotics are among the most influential global public health successes, selective pressures imposed by them drive bacterial genomic evolution. Spain^{23F}-1 is an excellent example of a bacterium that has become resistant to multiple antibiotics and that has evolved to become very successful in colonization, transmission, and causing disease. Moreover, Spain^{23F}-1 has subsequently shared its successful DNA with other unrelated pneumococci[72].

On the other hand, Spain^{9V}-3 belongs to ST156 (CC156), which, according to the MLST database, has been associated with a considerable diversity of capsular types (14, 9V, 19F, 11A, 9A, 15C, 13, 19A, and 15B), suggesting a high tendency of this clonal cluster to undergo capsular switching events. CC156, one of the largest CC presently found in the MLST database with frequent occurrence around the world[63], including Latin America and Brazil[61,73,74], is globally and consistently associated with important resistance profiles, including non-susceptibility to penicillin[75].

As PCV7 has been widely implemented worldwide, it is expected that these traditional resistant clones will lose ground because of selective pressure, given advantages to other clones/serotypes. A classic example of this natural biological event was the emergence of serotype 19A in both carriage and invasive disease soon after PCV7 implementation in the USA[76]. Of note, some clones of serotype 19A are consistently non-susceptible to penicillin, as well as resistant to other antimicrobials[77]. As a consequence, this capsular switching event dramatically increased the occurrence of penicillin non-susceptible isolates in many different regions of the world.

However, serotype 19A also increased in regions without vaccine selective pressure, suggesting the participation of other factors, such as temporal variations, dissemination of some specific clones, and antimicrobial pressure[78]. Four PMEN international clones are related to serotype 19A: Hungary^{19A}-6 (ST268), S.Africa^{19A}-7 (ST75), CSR^{19A}-1 (ST175), and S.Africa^{19A}-13 (ST41).

All but one (S.Africa[19A]-7) are multiresistant, including non-susceptible to penicillin (meningitis breakpoints).

Besides those above-mentioned clones, genotypic characterization of serotype 19A isolates by MLST showed that there are five major CC associated with them: CC81, CC193, CC199, CC276, and CC320[78]. ST320 (CC320), derived from Taiwan[19F]-14 (ST236) by capsular switching events, has become prevalent in many countries, and is strongly related to penicillin resistance[76,80-83]. Recently, it was observed that the genetic background of ST320 provides advantages associated with improved colonization in the nasopharynx when compared to ST199[77], another well-established serotype 19A clone, prevalent in the USA previously to PCV7. Indeed, this advantage may be responsible for the rapid shift of ST199 to ST320 in the USA soon after the introduction of PCV7[76].

As mentioned above, along with those antibiotic-resistant clones, PMEN also focus on some important disease-causing susceptible clones, such as the following related to serotypes included in one of the conjugate pneumococcal vaccines: Sweden[1]-27 (ST217), Sweden[1]-40 (ST304), Netherlands[3]-31 (ST180), Sweden[4]-38 (ST205), Portugal[6A]-41 (ST327), S.Africa[6B]-8(ST185), Netherlands[7F]-39 (ST191), and Colombia[23F]-26 (ST338).

Serotype 1 ranks among the most prevalent invasive serotypes in many countries[84-87] causing severe episodes of pneumonia and empyema in children[88]. In Brazil, since 1977, serotype 1 has been identified as one of the most frequent pneumococci causing severe infections in both adult and pediatric patients[89].

Some specific features are responsible for the epidemiological relevance of serotype 1, despite its susceptibility to most antimicrobials. First, a low colonization frequency, even in populations in which serotype 1 is a frequent cause of pneumococcal infections[90,91]. In addition, serotype 1 has the ability to cause outbreaks in communities and in crowded and closed institutions[92]. Besides, serotype 1 markedly presents low genetic diversity among the isolates, which has been associated with the short duration of carriage and/or a low density of this serotype in the nasopharynx, resulting in a reduced opportunity to exchange genes between strains[93].

Another serotype with high invasiveness power is serotype 3, which has been related with increased mortality in different regions[94-96]. Considering its genetic background, strains of serotype 3 belonging to ST180 have been associated with significant mortality[97]. Therefore, the high frequency of isolation of this serotype/ST and its relation with mortality needs continued surveillance to monitor for increases in this serotype post-PCV10 as this data may be important to consider the use of PCV13 in some regions.

Although somewhat controversial, serotype 7F also appears to be associated with high case-fatality[95]. Some authors have observed serotype 7F as one of the main serotypes associated with replacement following PCV7 introduction, through clonal expansion[98,99]. Pichon et al. demonstrated ST191 (serotype 7F) as the most prevalent clone causing meningitis 3 years after the introduction of PCV7 in England and Wales[99]. From reported studies, serotype 7F seems to be very rare in the nasopharynx of Brazilian children[59,73,90]. Besides, as it is part of the currently available pneumococcal vaccine (PCV10), it may not represent a worrisome occurrence in Brazil.

PCV7 was introduced in the United States in 2000, when almost half of all IPD was caused by pneumococci resistant to penicillin and/or macrolides[100]. As expected, following the introduction of pneumococcal vaccination, there was a substantial reduction in penicillin non-susceptible pneumococci occurrence[101]. However, subsequently to PCV7 usage, there has been an increase in pneumococcal disease due to non-PCV7 type pneumococci[102], many of which are now also penicillin non-susceptible, such as 19A and 6A, that are part of other vaccine formula, as well as other serotypes absent in any pneumococcal vaccine so far[103]. Therefore, despite the unquestionable beneficial effects of vaccination, the problem of resistance among pneumococci is far from solved.

In this context, eight PMEN clones include strains with serotypes not present in any of the currently available vaccine formula. They are related to the following STs: ST53 (Netherlands[8]-*33),* ST63 (Sweden[15A]-25), ST193 (Greece[21]-30), ST199 (Netherlands[15B]-*37),* ST218 (Denmark[12F]-34), ST377 (Utah[35B]-24), ST448 (USA[NT]-43), and ST344 (Norway[NT]-42). In general, they are multi-susceptible.

Some molecular characteristics of the Netherlands[8]-33 may explain its well-succeeded clonal spread: Jefferies et al. identified a pneumolysin allele 5 in ST53, a common worldwide-distributed ST related to serotype 8, that could facilitate the clonal expansion of those strains[104]. Besides, some authors include serotype 8 into the group of more invasive and/or the ones related to the worst outcomes. Therefore, as it

may become an important serotype in the post-vaccination era, and as antimicrobial usage may stimulate resistance occurrence, surveillance of these widely distributed serotype 8 clones is a subject of major concern.

Grabenstein et al. performed a systematic review to characterize differences in serious outcomes between pneumococcal serotypes. The authors show that serotype 8 was consistently related to an increase in severity of the disease, as well as serotype 15B[105]. The Netherlands^{15B}-37 is part of ST199, which also encompasses serotype 19A (among others), strongly related to penicillin resistance. The occurrence of the same genetic background (ST199) between serotype 19A and serogroup 15 is indicative of capsular switching.

Sweden^{15A}-25 belongs to ST63, which is worldwide distributed, including Latin America. This ST is essentially related to serotypes 15A, 14, 19F, and 19A. The capsular type 15A strain was found to only differ from the fully sequenced 19F clinical isolate G54 in the chemical composition of the capsular polysaccharide indicating that this lineage has the capacity to undergo *in vivo* capsular switch. A capsular switch may produce "vaccine escape" recombinants[94] that can avoid the vaccine-induced immune pressure, allowing pneumococcal survival as a species.

Of note, the serotype 19A (ST276) and 15A (ST63) clones have been identified as the *S. pneumoniae* clonal types most frequently recovered from pneumococcal infections in countries that introduced the PCV7 vaccine[80,106,107]. For unknown reasons, representatives of the third major colonizing clone with serotype 6A (ST2191) have not been recovered from pneumococcal disease. In contrast, colonization by each of the three major non-PCV7 clonal lineages has been widely reported[108].

Although Utah^{35B}-24 (ST377) presents a susceptible phenotype (albeit resistance to penicillin, considering meningitis CLSI criteria – MIC 1µg/mL), some post-vaccine works have demonstrated a relationship between this serotype and resistant profiles[109], as well as increased occurrence of this serotypes (and others such as 15A and 15B) in both carriage and invasive disease. Surveillance of serogroup 15, considering dissemination and resistance, may be of great relevance to the development of new vaccine formulations.

Similarly for serotype 8, serotype 12F has demonstrated increased occurrence after vaccination in some regions. Besides, it has been shown to cause outbreaks in human populations with identifiable risk factors[110]. This serotype has a high case/carriage ratio (CCR), i.e., it is a hyper invasive serotype, rarely found in nasopharynx[73,110]. One could expect that, after vaccine selective pressure, the pneumococcal population is supposed to suffer considerable changes, which may affect the behavior of such non-vaccine serotypes.

Based on the above, it is reasonable to conclude that the pneumococcal population is constantly changing, either because of biologically expected temporal changes or due to selective pressure exerted by vaccination and antibiotics. This situation may significantly alter the occurrence of antimicrobial resistance, and, in this context, epidemiological surveillance is consistently required to understand and monitor these changes, as they may directly affect patient care, as discussed below.

CONCLUSION

Pneumococcal infections are treated empirically. Limitations in the diagnostic methods, together with the severity of disease contribute to this procedure. Surveillance studies are crucial to define the prevalence of resistant strains both globally and in a particular region. Data obtained from such studies are generated by culture-dependent methods. Although different clones of PNSP are internationally distributed, and considering diseases other than meningitis, the prevalence to penicillin is quite low, making this old, safe, and inexpensive drug an attractive first choice to treat pneumococcal infections. The widespread use of conjugate vaccines among children, influencing the circulation of resistant clones, reinforces the need of surveillance studies to define the prevalence of resistance.

Finally, it is important to consider that almost all that is known about pneumococcal resistance comes from culture-insensitive methods. Culture independent methods are, in a certain sense, modifying some concepts about pneumococcal disease and once applied to the detection of resistant strains, they may also contribute to a better knowledge about resistance in pneumococci.

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