UNIVERSIDADE FEDERAL DA BAHIA
FACULDADE DE MEDICINA DA BAHIA
PROGRAMA DE PÓS-GRADUAÇÃO
EM CIÊNCIAS DA SAÚDE

COLONIZAÇÃO NASOFARÍNGEA E PNEUMONIA ADQUIRIDA NA COMUNIDADE CAUSADA POR *STREPTOCOCCUS PNEUMONIAE* EM CRIANÇAS: DIAGNÓSTICO E EFEITO DA VACINAÇÃO

Dafne Carvalho Andrade

Tese de Doutorado

Salvador (Bahia), 2017

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"Genius is one percent inspiration, ninety-nine percent perspiration"

Thomas Edison, 1932

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ÍNDICE

Lista de abreviações

Índice de tabelas e figuras

I. RESUMO 8

II. OBJETIVOS 10

III. INTRODUÇÃO 11

1. Pneumonia adquirida na comunidade 11

1.1 Diagnóstico etiológico 12

1.1.1. Papel da sorologia 14

1.1.1.1. Antígenos protéicos 15

1.1.2. Testes de avidez 17

1.2. Papel do Raio X de tórax 17

2. Colonização nasofaríngea 18

2.1. Efeito da vacinação pneumocócica na colonização nasofaríngea 19

3. Efeito da colonização e da infecção em dados sorológicos 20

3.1. Efeito da vacinação pneumocócica em dados sorológicos 22

IV. Artigo 1 (Serological diagnosis of pneumococcal infection in children with pneumonia using protein antigens: A study of cut-offs with positive and negative controls) 23

Journal of Immunolgical Methods 433:31-7, 2016.

V. Artigo 2 (Determination of avidity of IgG against protein antigens from *Streptococcus pneumoniae:* assay development and preliminary application in clinical settings) 31

Aceito para publicação em European Journal of Clinical Microbiology and Infectious Disease

VI. Artigo 3 (Infection by *Streptococcus pneumoniae* in children with or without radiologically confirmed pneumonia) 45

Aceito para publicação em Jornal de Pediatria (Rio de Janeiro)

VII. Artigo 4 (10-valent pneumococcal conjugate vaccine (PCV10) decreases metabolic activity but not nasopharyngeal carriage of *Streptococcus pneumoniae* and *Haemophilus influenzae*)..... 54

Vaccine, 35:4105-11, 2017

VIII. Artigo 5 (Antibody responses against *Streptococcus pneumoniae, Haemophilus influenzae* and *Moraxella catarrhalis* in children with acute respiratory infection with or without nasopharyngeal bacterial carriage) 63

Aceito para publicação em Infectious Diseases

IX. Artigo 6 (Effect of Pneumococcal Conjugate Vaccine on the Natural Antibodies and Antibody Responses Against Protein Antigens From *Streptococcus pneumoniae, Haemophilus influenzae* and *Moraxella catarrhalis* in children With Community-acquired Pneumonia)..... 73

Pediatric Infectious Disease Journal 35:683-9, 2016

X. DISCUSSÃO 81

1. Validação de um teste sorológico em multiplex com antígenos protéicos de *S. pneumoniae*.....81

2. Desenvolvimento e validação de um teste de avidez em multiplex com antígenos protéicos de *S. pneumoniae*.....86

3. Papel do Raio X de tórax no diagnóstico de PAC 90

4. Efeito da vacinação pneumocócica na colonização nasofaríngea por *S. pneumoniae, S. aureus, H. influenzae* and *M. catarrhalis* 96

5. Efeito da vacinação pneumocócica na detecção de respostas sorológicas e desenvolvimento de complicações entre crianças com IRA 99

6. Efeito da vacinação pneumocócica nos níveis basais de anticorpos e na frequência de resposta sorológica contra antígenos protéicos em crianças com PAC 102

7. Considerações finais 106

XI. CONCLUSÕES. 109

XII. PERSPECTIVAS DE ESTUDO.....111

XIII. SUMMARY 114

XIV. REFERÊNCIAS BIBLIOGRÁFICAS..116

ÍNDICE DE TABELAS E FIGURAS

I. Artigo 1 (Serological diagnosis of pneumococcal infection in children with pneumonia using protein antigens: A study of cut-offs with positive and negative controls) 23

Journal of Immunolgical Methods 433:31-7, 2016.

1. Tabela 1 26

2. Figura 1 27

3. Tabela 2 28

4. Tabela 3 28

5. Tabela 4 29

II. Artigo 2 (Determination of avidity of IgG against protein antigens from *Streptococcus pneumoniae:* assay development and preliminary application in clinical settings) 31

Aceito para publicação em European Journal of Clinical Microbiology and Infectious Disease

1. Figura 1 34

2. Figura 2 35

3. Tabela 1 36

4. Tabela 2 37

5. Figura 3 37

6. Tabela 3 38

7. Tabela 4 39

8. Tabela 5 40

9. Figura 4 41

10. Figura 5 41

11. Figura 6 42

III. Artigo 3 (Infection by *Streptococcus pneumoniae* in children with or without radiologically confirmed pneumonia) 45

Aceito para publicação em Jornal de Pediatria (Rio de Janeiro)

1. Figura 1 49

2. Tabela 1.....50

3. Tabela 2.....50

4. Tabela 3.....50

IV. Artigo 4 (10-valent pneumococcal conjugate vaccine (PCV10) decreases metabolic activity but not nasopharyngeal carriage of *Streptococcus pneumoniae* and *Haemophilus influenzae*)..... 54

Vaccine, 35:4105-11, 2017

1. Figura 1 57

2. Figura 2 58

3. Tabela 2.....58

4. Tabela 3.....58

5. Figura 3 59

6. Tabela 3.....59

7. Figura 3 60

8. Figura Suplementar 1.....62

VIII. Artigo 5(Antibody responses against *Streptococcus pneumoniae, Haemophilus influenzae* and *Moraxella catarrhalis* in children with acute respiratory infection with or without nasopharyngeal bacterial carriage) 63

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1. Figura 1 67

2. Tabela 1.....68

3. Tabela 2.....68

4. Tabela 3.....69

5. Tabela 4.....69

6. Tabela 5.....69

7. Tabela 6.....69

IX. Artigo 6 (Effect of Pneumococcal Conjugate Vaccine on the Natural Antibodies and Antibody Responses Against Protein Antigens From *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis* in children With Community-acquired Pneumonia) 73

Pediatric Infectious Disease Journal 35:683-9, 2016

1. Tabela 1.....76

2. Tabela 2.....77

3. Figura 1 77

4. Tabela 3.....78

5. Tabela 4.....78

LISTA DE ABREVIAÇÕES

CbpA: “Choline Binding Protein A”

DPI: Doença pneumocócica invasiva

IRA: Infecção respiratória aguda

OMA: Otite média aguda

PAC: Pneumonia adquirida na comunidade

PcpA: “*pneumococcal choline binding protein A*”

PcsB: “*protein required for cell wall separation of group B streptococcus*”

PCV: “*Pneumococcal conjugated vaccine*”/ Vacina pneumocócica conjugada

PhtD: “*pneumococcal histidine triad protein D*”

Ply: Pneumolisina

PspA: “*pneumococcal surface protein A*”

StkP-C: “*serine/threonine protein kinase*”

I. RESUMO

Pneumonia adquirida na comunidade (PAC) é uma das Infecções Respiratórias Agudas mais comuns na infância, apresentando altas taxas de morbi-mortalidade. Nós realizamos uma análise abrangente de fatores associados ao diagnóstico de infecção por *Streptococcus pneumoniae, Haemophilus influenzae* e *Moraxella catarrhalis* em crianças com PAC. Inicialmente, validamos um teste sorológico em multiplex com oito antígenos protéicos de *S. pneumoniae* (Ply, CbpA, PspA 1 e 2, PcpA, PhtD, StkP e PcsB) usando controles positivos e negativos. Este teste sorológico foi altamente sensível e específico para o diagnóstico de doença pneumocócica invasiva, e um aumento ≥ 2 vezes no nível basal de anticorpos foi um ponto de corte adequado para diagnóstico utilizando todas as proteínas testadas. Em seguida, desenvolvemos um teste de avidez utilizando proteínas pneumocócicas, o qual foi capaz de diferenciar com segurança crianças com e sem doença pneumocócica. Avaliamos também o papel do raio-X de tórax como um teste diagnóstico clínico para crianças com PAC que tiveram amostras sorológicas testadas para infecção por *S. pneumoniae, H. influenzae* ou *M. catarrhalis.* Crianças com pneumonia radiologicamente confirmada apresentaram maior taxa de infecção por *S. pneumoniae* quando comparadas com crianças com raio-X de tórax normal. Além disso, a presença de raio-X de tórax normal apresentou alto valor preditivo negativo para o diagnóstico de infecção por *S. pneumoniae.* O efeito do uso de vacina anti-pneumocócica em dados sorológicos também foi avaliado. Não foram encontradas diferenças na taxa de resposta sorológica a *S. pneumoniae, H. influenzae* ou *M. catarrhalis* em crianças que receberam ou não a vacina pneumocócica conjugada 10-valente (PCV10). No entanto, crianças vacinadas apresentaram níveis mais baixos de IgG contra antígenos protéicos de *S. pneumoniae* que crianças não vacinadas.

Em seguida, avaliamos o papel de *S. pneumoniae, H. influenzae* e *M. catarrhalis* como colonizadores nasofaríngeos, um passo fundamental para o desenvolvimento de doença invasiva. Não encontramos associação entre colonização nasofaríngea e detecção de resposta sorológica contra essas bactérias. Ao avaliar o efeito do uso de PCV10 na flora nasofaríngea, percebemos que crianças vacinadas apresentam menor taxa metabólica do *S. pneumoniae* que crianças não vacinadas. Por fim, não foi encontrada diferença nas taxas de colonização pelas bactérias avaliadas.

II. OBJETIVOS

- GERAL: Avaliar o papel de *Streptococcus pneumoniae, Staphylococcus aureus, Haemophilus influenzae* e *Moraxella catarrhalis* na colonização nasofaríngea e pneumonia adquirida na comunidade em crianças

- SECUNDÁRIOS:

- Desenvolver e validar um teste sorológico em multiplex utilizando antígenos protéicos de *S. pneumoniae* para diagnóstico de pneumonia comunitária em crianças
- Desenvolver e validar um teste de avidez utilizando antígenos protéicos para diagnóstico de infecção por *S. pneumoniae* em crianças
- Avaliar o papel do diagnóstico radiológico de pneumonia na determinação do agente etiológico em crianças com pneumonia adquirida na comunidade
- Avaliar o papel do uso da vacina pneumocócica conjugada 10-valente (PCV10) sobre o nível de anticorpos basais e frequência de resposta imune contra *S. pneumoniae, H influenzae* e *M. catarrhalis*
- Avaliar o efeito do uso de PCV10 sobre a frequência de colonização e perfil metabólico de *S. pneumoniae, S. aureus, H influenzae* e *M. catarrhalis* em crianças com infecção respiratória aguda
- Avaliar o efeito da colonização nasofaríngea por *S. pneumoniae, H influenzae* e *M. catarrhalis* na frequência de detecção de resposta de anticorpo contra estas bactérias.

III. INTRODUÇÃO

1. Pneumonia adquirida na comunidade

A pneumonia adquirida na comunidade (CAP) é uma das principais causas de morbidade e mortalidade na infância (Rudan et al., 2013; Walker et al., 2013, Liu et al., 2015), sendo responsável por 120 milhões de casos/ano (dos quais 14 milhões são casos graves) e 1 milhão de mortes em crianças menores de 5 anos de idade (Liu et al., 2015). Devido ao impacto da PAC pediátrica na saúde pública, foram implementadas estratégias de controle para alcançar o Objetivo de Desenvolvimento do Milênio 4 de "reduzir em dois terços, entre 1990 e 2015, a taxa de mortalidade dos menores de cinco anos" (WHO, 2009).

O manejo adequado de casos, com base na classificação de gravidade e seleção da estratégia terapêutica apropriada, é considerado elemento fundamental para controle da PAC (WHO, 2014). O diagnóstico de PAC é atualmente baseado na presença de sintomas respiratórios (como tosse ou dispnéia) associados à taquipnéia (WHO, 2014). Os casos de doença grave ou muito grave são identificados pela presença de retração subcostal e de sinais de gravidade (como dificuldade para beber líquidos, convulsões, letargia, estridor e cianose central), respectivamente (WHO, 2000; WHO, 2005). Exames laboratoriais ou radiológicos são recomendados apenas em casos com má evolução ou gravidade na admissão (Bradley, 2011; Harris, 2011).

Outras estratégias adotadas para reduzir a PAC pediátrica incluem controle de casos previníveis via vacinação e a prevenção e tratamento de condições debilitantes como infecção por HIV e desnutrição (WHO, 2009). A vacinação contra *S. pneumoniae* e *H. influenzae* tipo b já foi implementada no Brasil e gerou uma redução significativa no número de casos causados por estes agentes (O'Brien et al., 2009; Watt et al., 2009).

No entanto, existem vários desafios para controlar a PAC infantil. O uso de vacinas pneumocócicas baseadas em polissacarídeos capsulares reduziu a carga de colonização e doença por esses sorotipos, mas já foi relatado aumento na colonização por sorotipos não incluídos nas vacinas conjugadas (Dagan et al., 2009; Vesikari et al., 2016). Além disso, o diagnóstico etiológico da PAC continua a ser um desafio, uma vez que o diagnóstico por padrão-ouro depende do isolamento do agente etiológico de fluidos corporais estéreis, como sangue ou efusão pleural. Embora esta estratégia diagnóstica seja altamente específica, ela é pouco sensível, já que a minoria dos pacientes com PAC tem hemoculturas positivas (Shah et al., 2010) e o agente etiológico nem sempre pode ser isolado na análise de efusão pleural (Nascimento-Carvalho et al., 2013).

1.1. Diagnóstico etiológico

A PAC pediátrica pode ser causada por uma ampla gama de agentes bacterianos e virais. A PAC causada por vírus já foi relatada em até 15-40% dos casos (Rudan et al., 2008), incluindo doença causada pelo vírus sincicial respiratório (RSV), influenza e parainfluenza, adenovírus, rinovírus (Harris, 2011 et al., Esposito et al., 2013) e outros vírus incomuns, como o metapneumovírus (Nascimento-Carvalho et al., 2011) e o bocavírus (Nascimento-Carvalho et al., 2012). Uma recente revisão de literatura demonstrou a importância de agentes virais dentre os casos de PAC infantil, estimando que o VSR seja atualmente o agente mais comum de PAC, responsável por 28,8% de todos os episódios, seguido do vírus influenza, que representou 17% dos casos (Rudan, 2013).

A avaliação do papel dos agentes bacterianos como causa da PAC é mais complicada, uma vez que as incidências variam de acordo com os critérios aplicados para o diagnóstico de PAC e com o método diagnóstico aplicado para a detecção de patógenos.

Além disso, a maioria dos dados disponíveis sobre agentes bacterianos de PAC são provenientes de ensaios clínicos avaliando o efeito de vacinas, a partir dos quais dados etiológicos são extraídos como uma observação indireta da redução de casos de pneumonia após a vacinação (Rudan et al., 2008). *S. pneumoniae* e *H. influenzae* são as bactérias que mais comumente causam PAC, seguidos por *S. aureus, M catarrhalis* e bactérias gram negativas (Rudan et al., 2008; Harris et al., 2011; Juven et al., 2000; Nascimento-Carvalho, 2001). Dados de uma recente revisão de literatura de fato demonstraram que *S. pneumoniae* e *H. influenzae* são as principais causas bacterianas da PAC, responsáveis por 6.9 e 2.8% de todos os casos de PAC infantil em 2010, respectivamente (Rudan et al., 2013). Bactérias atípicas também podem causar PAC infantil, sobretudo *Mycoplasma pneumoniae* e *Chlamydophila pneumoniae* (Harris et al., 2001; Nascimento Carvalho, 2001). Além disso, dados recentes indicam que cerca de 1% dos casos de PAC grave ou muito grave podem ser causados por *Bordetella pertussis* (Barger-Kamate et al., 2016)

A etiologia da PAC, no entanto, varia com a gravidade da doença. Rudan *et al*, (2013), relataram um aumento das taxas de detecção de *S. pneumoniae* e *H. influenzae* e diminuição da detecção de VSR e influenza em crianças com PAC grave e uma proporção ainda maior de causas bacterianas de PAC foi relatada entre casos de PAC que evoluíram ao óbito (Rudan et al., 2013). Da mesma forma, Nascimento-Carvalho *et al* (2016) encontraram um aumento progressivo no número de casos causados por infecção bacteriana em crianças com PAC não grave, grave ou muito grave (12,5% versus 29,3% vs. 55,6%, respectivamente; p: 0,04). A etiologia da PAC também é influenciada por outros fatores, como idade, vacinação, estado nutricional e presença de comorbidades (Harris et al., 2011).

Em relação às técnicas disponíveis para o diagnóstico etiológico de PAC, o padrão-ouro ainda é o isolamento do agente em líquidos corporais estéreis, o que é pouco sensível. Os testes moleculares são promissores, com boa especificidade e potencial de uso em multiplex. No entanto, esses testes requerem um arsenal tecnológico que não está habitualmente disponível. Além disso, o alto custo, a necessidade de padronização e complexidade do procedimento limitam a aplicação do diagnóstico molecular atualmente (Bhat et al., 2012). Neste contexto, as técnicas sorológicas ressaem como técnicas adequadas para o diagnóstico etiológico da PAC em estudos epidemiológicos.

1.1.1. Papel da sorologia

Os testes sorológicos baseiam-se na detecção de anticorpos (geralmente IgG) contra componentes dos patógenos estudados (Korppi et al., 2008a). A detecção de um aumento de IgG entre amostras coletadas em um intervalo de tempo predeterminado serve como evidência indireta da ativação do sistema imune causada pela infecção pelo patógeno estudado. Até o momento, a maioria dos testes sorológicos para a detecção de agentes bacterianos de PAC utiliza antígenos polissacarídicos ou "*whole cell antigen*".

O método mais comumente utilizado para a quantificação de anticorpos é o ELISA, que é reconhecidamente específico e sensível (Korppi et al., 2008a). No entanto, quando a avaliação da resposta imune a múltiplos antígenos é necessária, o ELISA torna-se demorado e caro. Além disso, como a análise é limitada a um antígeno por teste, um grande volume de soro seria necessário para realizar testes contra múltiplos antígenos, o que geralmente é difícil de obter ao lidar com pacientes pediátricos. Neste contexto, o uso de um teste multiplex permitiria a detecção de anticorpos contra vários antígenos simultaneamente, reduzindo a quantidade de soro necessária e gerando grande quantidade de dados.

O diagnóstico sorológico de infecção por *S. pneumoniae* geralmente é feito pela detecção de um aumento nos títulos de anticorpos a polissacarídeos capsulares, o que permite a discriminação do sorotipo infectante. O ponto de corte para a detecção da resposta sorológica foi estabelecido como um aumento de 2 vezes nos níveis basais de anticorpos quando utilizando estes antígenos (Korppi et al., 2008a). O uso de polissacarídeos capsulares permite a detecção de respostas específicas contra *S. pneumoniae*, mas limita a sensibilidade do método, pois as respostas contra sorotipos não incluídos no teste não são detectadas. Testes sorológicos utilizando o polissacarídeo C de *S pneumoniae* também foram validados, sendo selecionado um aumento de 3 vezes nos níveis de anticorpos basais como ponto de corte para diagnóstico de infecção pneumocócica.

Testes sorológicos para *H. influenzae* e *M. catarrhalis* para a detecção do agente etiológico de PAC foram realizados utilizando "*whole cell antigen*", e um aumento de 3 vezes no nível de anticorpos basais foi utilizado como ponto de corte (Nohynek et al., 1995). No entanto, o uso de "*whole cell antigen*" também apresenta limitações, uma vez que a inclusão de vários antígenos celulares pode limitar a especificidade da reação.

Neste cenário, novos antígenos protéicos de *S. pneumoniae, H. influenzae* e *M. catarrhalis* foram recentemente identificados e reconhecidos como antígenos conservados e específicos para cada espécie (Tai, 2006; Poolman et al., 2000; Murphy & Parameswaran, 2009; Principi & Esposito, 2011). Esses antígenos são atualmente considerados candidatos para inclusão em formulações vacinais e testes sorológicos (Andrade et al., 2014), permitindo a detecção de resposta sorológica a antígenos específicos para cada espécie sem limitação de sorotipo.

1.1.1.1. Antígenos protéicos

Várias proteínas pneumocócicas foram bem caracterizada até o momento e estão sendo consideradas para uso em testes sorológicos e desenvolvimento de vacinas (Principi, 2011). Entre elas, pneumolisina (Ply), "*choline binding protein A*" (CbpA), "*pneumococcal surface protein A*" (PspA), "*pneumococcal choline binding protein A*" (PcpA), "*pneumococcal histidine triad protein D*" (PhtD), "*serine/threonine protein kinase*" (StkP) e "*protein required for cell wall separation of group B streptococcus*" (PcsB) são antígenos promissores para uso em testes sorológicos. Ply é uma citotoxina altamente conservada liberada durante a autólise que interage com o sistema imune do hospedeiro de múltiplos modos (Tai, 2006; van der Poll & Opal, 2009). CbpA (Brooks-Walter et al., 1999; Tai, 2006; van der Poll & Opal, 2009) e PspA (Crain et al., 1990; Briles et al., 2000; Tai, 2006; van der Poll & Opal, 2009; Croney et al., 2012) também estão presentes na maioria das cepas de *S. pneumoniae* e desempenham papéis no desenvolvimento de infecção por este agente. PcpA é uma proteína de ligação de colina presente na maioria das cepas virulentas de *S. pneumoniae* e está envolvida na adesão da bactéria a células epiteliais (Khan et al., 2012a). Da mesma forma, PhtD é uma proteína de superfície altamente conservada da família Pht (Adamou et al., 2001), que recentemente foi reconhecida como adesina (Khan & Pichichero, 2012b). StkP e PcsB são proteínas imunogênicas recentemente descobertas, que devem desempenhar papéis na divisão celular e no metabolismo de peptidoglicanos, respectivamente (Giefing et al., 2007; Giefing et al., 2010; Giefing-Kroll et al., 2011).

Entre os antígenos protéicos de *H. influenzae* descritos até o momento, a Proteína D é um fator de virulência conservado presente em todas as cepas desta bactéria (Poolman et al., 2000) e que atualmente é utilizado como carreador na vacina pneumocócica conjugada 10-valente (PCV10). Várias adesinas de *M. catarrhalis* que seriam adequadas para uso em testes diagnósticos já foram identificadas, como a "Outer Membrane Protein

CD" (OMP CD), uma adesina que também possui funções na patogênese desta bactéria, e Msp22, uma lipoproteína de superfície (Murphy & Parameswaran, 2009 Saito et al., 2013; Smidt et al., 2013).

1.1.2. Testes de avidez

O teste de avidez representa uma alternativa para avaliar a resposta imune sem a necessidade de amostras pareadas de soro. A avidez representa a força da ligação entre o antígeno e o anticorpo e reflete a qualidade da resposta imune (Fried, 2013). De fato, uma correlação negativa entre a avidez do anticorpo estudado e a concentração de anticorpos necessária para a atividade opsonofagocítica/bactericida foi relatada em estudos anteriores (Schlesinger & Granoff, 1992; Antilla et al., 1999; Usinger & Lucas, 1999).

Os testes de avidez já são utilizados com sucesso como ferramenta de diagnóstico para infecções virais (Meriluoto et al., 2012; Chen et al., 2014). Quanto ao uso da avidez contra os agentes bacterianos, a avidez da IgG contra os polissacarídeos pneumocócicos também foi amplamente estudada nos ensaios de vacinas, com aumentos na avidez de IgG após a vacinação pneumocócica (Ekstrom et al., 2007; Ekstrom et al., 2013). Além disso, dados recentes relataram aumentos na avidez de IgG após exposição ao *S. pneumoniae* (Ota et al., 2011; Fried et al., 2013). Até o momento, a avidez de IgG contra as proteínas pneumocócicas só foi avaliada em poucos estudos. Dentre esses, destaca-se a avaliação de uma vacina experimental utilizando StkP, PcsB, PspA e "pneumococcal surface adhesin A" (PsaA), em que houve aumento da avidez após a vacinação (Olafsdottir et al., 2012).

1.2. O raio-X de tórax

O raio-X de tórax pode auxiliar no diagnóstico da PAC pediátrica através da identificação de padrões radiológicos sugestivos de um processo inflamatório, como

infiltrados pulmonares, por exemplo. No entanto, existem limitações importantes para o uso rotineiro deste exame, como uma baixa concordância entre observadores (Johnson et al., 2010) e a incapacidade de distinguir entre agentes etiológicos específicos (Korppi et al., 2008b; Don et al., 2009). Além disso, quando o impacto da realização de um raio-X de tórax sobre a evolução das crianças com PAC foi avaliado, nenhum benefício foi descrito (Swingler et al., 1998). Conseqüentemente, as diretrizes atuais para o manejo da PAC segundo a Sociedade Britânica de Tórax ("British Thoracic Society"), a Sociedade Americana de Doenças Infecciosas e Sociedade de Doenças Infecciosas Pediátricas ("Pediatric Infeccious Diseases Society" e "Infectious Diseases Society of America") não recomendam mais a realização rotineira de um raio-X de tórax diante de um caso com suspeita de PAC (Bradley et al., 2011; Harris et al., 2011)

Por outro lado, uma proporção significativa de crianças com diagnóstico clínico de PAC apresenta uma radiografia de tórax normal à admissão (Hazir et al, 2006; Xavier-Souza et al, 2013). Além disso, crianças que apresentam ou não PAC radiologicamente confirmada são diferentes na admissão e também evoluem de forma diferente (Cardoso et al, 2011; Key et al, 2011; Fontoura et al, 2012). Um efeito diferencial da vacinação pneumocócica também foi descrito dependendo dos critérios diagnósticos da PAC, com maior redução proporcional no número de casos de PAC em crianças com confirmação radiológica (Lucero et al, 2009; Tregnaghi et al, 2014). Em conjunto, esses dados sugerem que a doença em crianças com diagnóstico clínico de PAC com ou sem confirmação radiológica podem ter mecanismos de doença distintos e diferentes agentes etiológicos.

2. Colonização nasofaríngea

A colonização nasofaríngea é um processo dinâmico no qual as bactérias comensais e potencialmente patogênicas são constantemente adquiridas e eliminadas da nasofaringe. Este processo é afetado por vários fatores, incluindo idade, colonização simultânea por outras bactérias (Patel et al, 2015), vacinação (Vesikari et al, 2016) e frequência a creches (Verhaegh et al, 2010). A colonização por bactérias patogênicas é considerada um passo fundamental para o desenvolvimento de doença invasiva por estes agentes (Laval et al, 2006; Simell et al, 2012; Tenebaum et al, 2012). Além disso, a presença de colonização por *S. pneumoniae* em alta densidade em pacientes com infecção respiratória aguda (IRA) tem sido associada ao desenvolvimento de doença pneumocócica invasiva (Wolter et al, 2014). Além disso, a colonização nasofaríngea bacteriana também afeta características clínicas em crianças com IRA viral (Yu et al, 2009, Jartti et al, 2011), sendo associada a maior duração de hospitalização e maior risco de sibilância recorrente em crianças colonizadas (Jartti et al, 2011).

S. pneumoniae, H. influenzae, M. catarrhalis e *S. aureus* são bactérias que colonizam frequentemente a nasofaringe de crianças e apresentam complexas interações entre si (van den Bergh et al, 2012; Xu et al, 2012). Associações positivas foram descritas entre colonização por *S. pneumoniae* e *H. influenzae* (van den Bergh et al, 2012) e *M. catarrhalis* (van den Bergh et al, 2012; Xu et al, 2012). Além disso, associações negativas foram encontradas entre colonização por *S. aureus* e *S. pneumoniae* (van den Bergh et al, 2012; Xu et al, 2012) e entre *S. aureus* e *M. catarrhalis* (Xu et al, 2012).

2.1. Efeito da vacinação pneumocócica sobre colonização nasofaríngea

A vacinação pneumocócica promove a diminuição da colonização pelos sorotipos de *S. pneumoniae* incluídos na vacina e aumento dos sorotipos não incluídos na mesma (Brandileone et al, 2016, Vesikari et al, 2016), o que tem como resultado uma freqüência

geral inalterada na colonização pelo *S. pneumoniae* (Lindstrand et al, 2016). Em relação à colonização por *H. influenzae*, Camili et al. (2015) demonstraram um aumento na colonização por esta bactéria entre as crianças que foram vacinadas com PCV7 e com PCV13 (Camili et al, 2015). Da mesma forma, evidências recentes sugerem que vacinação com PCV10 não reduz as taxas de colonização por *H. influenzae* (Brandileone et al, 2016; Vesikari et al, 2016), apesar da presença da proteína D do *H. Influenzae* na sua composição. Não houve diferença nas taxas de colonização por *M. catarrhalis* após a vacinação com PCV7 (van Gils et al, 2011) ou PCV10 (Vesikari et al, 2016). A colonização por *S. aureus* aumentou após a vacinação com PCV7 (Devine et al, 2015). No entanto, não foram encontradas alterações nas taxas de colonização por *S. aureus* em um ensaio clínico avaliando o efeito da PCV10 na colonização nasofaríngea de bactérias patogênicas em crianças (Vesikari et al, 2016).

3. Efeito da colonização e infecção por bactérias patogênicas em dados sorológicos

Poucos estudos abordaram o desenvolvimento de anticorpos contra antígenos protéicos de *S. pneumoniae, H. influenzae e M. catarrhalis* (Holmlund et al, 2009; Lebon et al, 2011; Prevaes et al, 2012; Vehaegh et al, 2012; Ditse et al, 2013; Borges et al, 2016). Holmlund et al (2009) descreveram que os níveis de anticorpos contra as proteínas pneumocócicas CbpA, PhtD e LytC foram semelhantes entre amostras de sangue do cordão umbilical e respectivas mulheres grávidas; além disso, os níveis de IgG apresentaram uma diminuição inicial durante as primeiras 18 semanas de vida (Holmlund et al, 2009). Borges et al (2016) relataram que, em uma coorte de 50 crianças finlandesas seguidas até 13 anos de vida, os níveis de anticorpos contra as proteínas pneumocócicas atingiram um pico aos 3 a 5 anos e, em seguida, estabilizaram-se; os níveis de anticorpos contra as proteínas de *H. influenzae* atingiram um pico durante o segundo ano de vida e depois estabilizaram-se; por fim, os níveis de anticorpos contra as proteínas de *M.*

catarrhalis atingiram seu pico durante o primeiro ano de vida e depois diminuíram lentamente (Borges et al, 2016). Estes dados estão de acordo com relatos prévios da literatura que demonstraram que a produção de anticorpos contra antígenos protéicos começa em diferentes idades para cada antígeno (Rapola et al, 2000; Holmlund et al, 2006; Zhang et al, 2006; Holmlund et al, 2009; Simell et al, 2009; Pichichero et al, 2010; Lebon et al, 2011; Prevaes et al, 2012; Hagerman et al, 2013)

Quanto ao efeito da colonização bacteriana em dados sorológicos, coortes prospectivas identificaram aumento nos níveis de anticorpos contra proteínas pneumocócicas após colonização por *S. pneumoniae* (Simell et al, 2009; Lebon et al, 2011; Hagerman et al, 2013). Da mesma forma, foram detectados aumentos nos níveis de IgG contra 5 antígenos protéicos de *M. catarrhalis* (OMP CD, Msp22, "*oligopeptide permease*"–Opp-A, "*hemagglutinin*" –Hag-, e "*PilA clade 2*" -PilA2) após colonização (Ren et al, 2015). O aumento da IgG para proteínas pneumocócicas induzida pela colonização por esta bactéria, no entanto, não foi grande o suficiente para permitir a detecção de uma resposta de anticorpos (definida como um aumento de 2 vezes nos níveis basais de anticorpos) em uma coorte de 36 crianças seguidas durante os primeiros 2 anos de vida (Turner et al, 2013). Além disso, níveis mais elevados de IgG a proteínas pneumocócicas não foram associados a uma redução na ocorrência de futuros episódios de colonização pneumocócica (Prevaes et al, 2012).

Aumentos nos níveis de anticorpos a proteínas pneumocócicas (avaliados como variável contínua) também foram relatados após a otite média aguda (OMA) (Rapola et al, 2001; Simell et al, 2009). No entanto, no contexto da OMA, também foram detectadas respostas sorológicas (definidas como um aumento de 2 vezes nos níveis de anticorpos) a antígenos protéicos de *S. pneumoniae* em casos de OMA causada por este agente (Rapola et al., 2001). Portanto, é esperado que em crianças com PAC causada por *S. pneumoniae,*

H. influenzae ou *M. catarrhalis*, respostas sorológicas a antígenos protéicos destas bactérias sejam detectadas, devido à natureza invasiva desta doença.

3.1. Efeito da vacinação pneumocócica em dados sorológicos

Prevaes et al (2012) relataram uma tendência para menores níveis de IgG em crianças vacinadas com PCV7 para os antígenos Nan, Pilus A, PspA e PsaA em um grupo de crianças menos de 24 meses (Prevaes et al., 2012). No entanto, Ditse et al (2009) não encontraram diferenças nos níveis de IgG contra 15 proteínas pneumocócicas entre crianças vacinadas ou não com PCV9 (Ditse et al., 2013). Portanto, o efeito da vacinação pneumocócica nos níveis de anticorpos basais e na detecção de resposta sorológica a antígenos protéicos de *S. pneumoniae* ainda precisa ser definido.

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IV. Artigo 1

SEROLOGICAL DIAGNOSIS OF PNEUMOCOCCAL INFECTION IN CHILDREN WITH PNEUMONIA USING PROTEIN ANTIGENS: A STUDY OF CUT-OFFS WITH POSITIVE AND NEGATIVE CONTROLS

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Research paper

Serological diagnosis of pneumococcal infection in children with pneumonia using protein antigens: A study of cut-offs with positive and negative controls

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ABSTRACT

The etiological diagnosis of infection by *Streptococcus pneumoniae* in children is difficult, and the use of indirect techniques is frequently warranted. We aimed to study the use of pneumococcal proteins for the serological diagnosis of pneumococcal infection in children with pneumonia. We analyzed paired serum samples from 13 Brazilian children with invasive pneumococcal pneumonia (positive control group) and 23 Finnish children with viral pharyngitis (negative control group), all aged <5 years-old. Children with pharyngitis were evaluated for oropharyngeal colonization, and none of them carried *S. pneumoniae*. We used a multiplex bead-based assay with eight proteins: Ply, CbpA, PspA1 and 2, PcpA, PhtD, StkP and PcsB. The optimal cut-off for increase in antibody level for the diagnosis of pneumococcal infection was determined for each antigen by ROC curve analysis. The positive control group had a significantly higher rate of ≥2-fold rise in antibody levels against all pneumococcal proteins, except Ply, compared to the negative controls. The cut-off of ≥2-fold increase in antibody levels was accurate for pneumococcal infection diagnosis for all investigated antigens. However, there was a substantial increase in the accuracy of the test with a cut-off of ≥1.52-fold rise in antibody levels for PcpA. When using the investigated protein antigens for the diagnosis of pneumococcal infection, the detection of response against at least one antigen was highly sensitive (92.31%) and specific (91.30%). The use of serology with pneumococcal proteins is a promising method for the diagnosis of pneumococcal infection in children with pneumonia. The use of a ≥2-fold increase cut-off is adequate for most pneumococcal proteins.

1. Introduction

Streptococcus pneumoniae infection is an important cause of morbidity and mortality in children, causing both invasive and non-invasive diseases (O'Brien et al., 2009). The diagnosis of infection by *S. pneumoniae* is difficult, particularly in children with community acquired pneumonia (CAP), as the gold standard for the etiological diagnosis is isolation of the bacterium from sterile tissues. This diagnostic strategy is highly specific, but poorly sensitive, because the minority of CAP patients has positive blood cultures for pneumococcus (Shah et al., 2010) and this bacterium cannot always be isolated in pleural effusion analysis (Nascimento-Carvalho et al., 2013). Furthermore, although molecular diagnostic tests are a promising method (De Schutter et al., 2014), they are not commonly available. In this scenario, indirect techniques have been developed to identify the cases of infection caused by *S. pneumoniae*.

Serological techniques are a suitable alternative for the diagnosis of invasive pneumococcal infection, especially in research or epidemiological surveillance. The rational for these tests relies on the microbe-specific immune responses elicited by the individual upon contact with *S. pneumoniae*. However, the use of serology in children has been criticized, mainly due to insufficient validation with bacteremic samples (Korppi et al., 2008). Furthermore, as many serological tests detect antibodies to serotype-specific polysaccharides, the sensitivity of such assays has been questioned. The inclusion of pneumococcal proteins in serological assays represents an option to improve the performance of the diagnostic tests, as proteins are more immunogenic than polysaccharides (Korppi et al., 2008). Also, many pneumococcal proteins are highly specific for *S. pneumoniae*, and have a ubiquitous distribution among the various pneumococcal strains (Tai, 2006).

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However, many pneumococcal proteins have only been recently described and identified as antigens, and they have not been validated for use in serology. Thus far, most studies have used the cut-off of ≥2-fold increase in antibody levels to recognize an antibody response. This cut-off was determined for pneumolysin (Ply), a pneumococcal cytotoxin, using data from a group of healthy children (Nohynek et al., 1995). However, there was no validation performed with true-positive controls. Scott et al. (2005) evaluated the use of pneumococcal surface adhesin A (PsaA) for serology in children with invasive pneumococcal disease (IPD) and healthy Kenyan children, and found that the cut-off of ≥2.7-fold increase in antibody levels against this antigen was adequate for pneumococcal infection diagnosis (Scott et al., 2005). Therefore, distinct protein antigens may have different thresholds for the detection of an antibody response when evaluated by distinct serological methods. We aimed to study the use of pneumococcal proteins for the serological diagnosis of pneumococcal infection in children with pneumonia using a multiplexed bead based assay.

2. Methods

2.1. Study participants

2.1.1. Positive controls

The positive control group comprised 13 Brazilian children 2–59 months old with invasive pneumococcal pneumonia who were enrolled in a prospective study for the etiological diagnosis of community-acquired pneumonia carried out at the Professor Hosannah de Oliveira Pediatric Centre, Federal University of Bahia, Salvador, Northeast Brazil, from September 2003 to May 2005 (Nascimento-Carvalho et al., 2008). All children were hospitalized and had serum samples collected at admission and 2–4 weeks later. None of the children were vaccinated against *S. pneumoniae*.

Invasive pneumococcal pneumonia was diagnosed by the isolation of *S. pneumoniae* on blood culture (9 patients) or by the detection of pneumococcal DNA in the buffy-coat (4 patients) using PCR with the *ply* primers. Data about this study group have been previously published elsewhere (Nascimento-Carvalho et al., 2008; Borges et al., 2015).

Blood culture testing was carried out at the Federal University of Bahia. From each of the collected blood specimens, 0.5–4.0 mL were immediately inoculated in 20 mL of supplemented BHI and incubated in OrganonBact/Alert equipment at 35 °C, for seven days. After a positive result was informed by the equipment, the inoculated medium was subjected to sub-culture on Columbia agar with 5% lamb blood and on agar-chocolate, and was again incubated for 18–24 h at 35 °C with 5% CO_2. *S. pneumoniae* was distinguished from other alpha-hemolytic streptococci by means of tests for solubility in bile and optochin. The identification of pneumococcal isolates was confirmed at Adolfo Lutz Institute (National Reference Laboratory, Brazilian Ministry of Health).PCR assays were carried out at the National Public Health Institute, Oulu, Finland. Extraction of DNA was performed using QIamp DNA Blood Mini-Kit (Qiagen, Hilden, Germany) (Nascimento-Carvalho et al., 2008).

2.1.2. Negative controls

The negative control group included 23 Finnish children 12–59 months old with pharyngitis evaluated at the Turku University Hospital, Turku, Finland, from January 2014 to February 2015. Children included in this study were previously healthy. At admission, all children had oropharyngeal samples collected for culture, and none presented current colonization by *S. pneumoniae*. Eighty-seven percent of children with pharyngitis had viral infection. Acute and convalescent blood samples were collected at admission and 2–5 weeks later. Data on pneumococcal vaccination were not collected; however, vaccination coverage with 10-valent pneumococcal conjugate vaccine has been estimated to be 95% after it was implemented in Finland in 2010 (http://www.thl.fi/roko/rokotusrekisteri/kattavuusraportit2014, n.d.).

2.2. Reagents

The presence of antibodies against *S. pneumoniae* was investigated using eight distinct pneumococcal proteins: pneumolysin [Ply], choline binding protein A [CbpA], pneumococcal surface protein A families 1 and 2 [PspA 1 and PspA2], pneumococcal choline binding protein A [PcpA], pneumococcal histidine triad protein D [PhtD], serine/threonine protein kinase [StkP-C, SP1732-3, a C-terminal fragment of StkP], and protein required for cell wall separation of group B streptococcus [PcsB-N, SP2216-1, a N-terminal fragment of PcsB]). Hydroxysulfosuccinimide (Sulfo-NHS) and 1-ethyl-3 (3-dimethylamino-propyl) carbodiimide-HCl (EDC) were provided by Thermo Fisher Scientific, Rockford, IL, USA. R-phycoerthyryn (R-PE)-conjugated AffiniPure goat anti-human IgG, Fc_γ Fragment specific was obtained from Jackson ImmunoResearch Laboratories Inc. (Westgrove, PA, USA).

2.3. Serology

2.3.1. Conjugation of pneumococcal proteins to the beads

The levels of antibodies against pneumococcal proteins were determined as described (Andrade et al., 2014) with a few modifications using a multiplexed bead based serological assay with Luminex xMAP® technology. Pneumococcal proteins were conjugated to Microplex beads using a 2-step carbodiimide reaction (Andrade et al., 2014), creating 7 distinct bead sets (one for each protein, except for PspA 1 and 2, which were conjugated together on the same bead). Beads were initially activated using a solution containing 5 mg/mL of Sulpho-NHS and EDC. After a 20-minute incubation, the beads were washed twice with phosphate buffered saline (PBS) and submitted to incubation with protein solution for 1.5 h in the dark. Subsequently, beads were washed twice with PBS and stored in a solution of PBS containing 10% fetal bovine serum (F-PBS) and 0.01% sodium azide.

2.3.2. Luminex assay

Sera were diluted 1:400 in F-PBS, and 25 μL of the diluted serum sample and 25 μL of bead solution were transferred to each well of a 96 well plate (Millipore MSHVN4510, Merck KGaA, Darmstadt, Germany). The mixture was then incubated for 1 h in the dark at 600 rpm. The plate was then washed with F-PBS using a vacuum washer, and 50 μL of a 1:100 solution of diluted RPE was added to each well. The plate was incubated for 30 min in the dark at 600 rpm and washed with F-PBS using a vacuum washer. Finally, 80 μL of F-PBS was added to each well (Andrade et al., 2014).

The pneumococcal reference serum 007 (Goldblatt et al., 2011) was included on each plate as a standard and was assigned an arbitrary antibody concentration of 1000 U/mL for each antigen. Patient sera antibody concentration was determined in relation to the amount of antibodies assigned in the 007 serum. Control sera with high and low antibody concentrations were analyzed on each plate to ensure good batch to batch consistency, and presented a coefficient of variation <20% for all proteins. Samples were assayed in duplicate and the results averaged. Acute and convalescent samples were always tested on the same plate. Samples were analyzed using a Luminex 200 (xMAP® Technology, Luminex Corporation, Austin, TX, USA) device. All tests were performed by DCA and ICB from October 2014 to February 2015. The samples used in this serological test were immediately processed after collection and were stored at −20 °C until the moment of analysis.

2.4. Ethical committee approval

The use of the samples from Brazilian children was approved by the Ethics Committee of the Federal University of Bahia, and the use of the samples from Finnish children was approved by the Ethics Committee of the Hospital District of Southwest Finland. All legal guardians provided written informed consent before enrolment in the investigation.

2.5. Statistical analysis

Categorical variables were presented as absolute number (percentage) and continuous variables as median (25th–75th percentiles) as they showed non-parametric distribution. Categorical variables were compared using chi-square or Fisher's exact test as appropriate and continuous variables were evaluated using Mann–Whitney U test. In order to determine the optimal parameters of antibody responses between the acute and convalescent serum samples for the diagnosis of pneumococcal infection, the sensitivity and specificity of different cut-offs were evaluated. Receiver operating characteristic (ROC) curves were plotted to determine cut-offs of fold-increase in antibody levels, using the ratio between acute and convalescent samples as the continuous variable and the origin of the sample (from either positive or negative controls) as the dichotomous variable. We determined the optimal cut-off points based on the accuracy of each cut-off. Sensitivity and specificity of the usual cut-off of ≥2-fold increase in antibody levels were also evaluated for each of the studied antigens for comparison with the cut-offs determined herein. The optimal number of antibody responses against different proteins needed for the diagnosis of invasive pneumococcal infection was also determined by ROC curve analysis. Logistic regression was performed using the presence of antibody response against each antigen considering the cut-off defined herein as the dependent variable, and the study group and the levels of the respective antibodies in the acute serum sample as independent variables. The software Stata (version 13.0) was used for the analysis.

3. Results

This study included 36 children whose median age was 28 months ([25th–75th percentile]: 15.25–42.5 months) and 54.1% were males. The median interval of serum sample collection was 19 days ([25th–75th percentile]: 16–24 days). The positive control group was younger than the negative controls (median [25th–75th percentile]: 14 [9.5–24.5] *vs.*37 [25–48] months; $P < 0.001$), and there was no statistical difference on serum sample collection interval between the two groups (median [25th–75th percentile]: 20 [16.5–25.5] *vs.*18 (Turner and Turner, 2013; Olaya-Abril et al., 2015; van der Poll and Opal, 2009; Giefing et al., 2008; Jiménez-Munguía et al., 2015; Holmlund et al., 2009; Lebon et al., 2011; Simell et al., 2009; Hagerman et al., 2013) days; $P = 0.6$) and on the gender (76.9% *vs.* 43.5% of males, $P = 0.052$). The baseline antibody levels were higher in the negative control group for Ply, CbpA, PcpA, PhtD and StkP-C(Table 1). Twelve out of the thirteen children in the positive control group had a ≥2-fold increase in antibody levels to at least one pneumococcal protein, while only two out of 23 children had ≥2-fold responses in the negative control group. The patient from the positive control group who failed to respond against *S. pneumoniae* had invasive disease diagnosed by PCR and was 28 months old. The two patients from the negative control group who responded against *S. pneumoniae* had other agents detected: one patient had Group A Streptococcus cultured from her throat; the second patient had infection by Epstein–Barr Virus (EBV) based on the detection of specific IgM. The significant higher rates of ≥2-fold antibody responses in the positive controls when compared to negative controls were seen against all the studied pneumococcal antigens, except Ply (Table 1).

The ROC curves for each of the studied pneumococcal antigens are shown in Fig. 1. Table 2 shows the area under the curve and optimal cut-offs for each antigen for the diagnosis of pneumococcal infection, as well as the sensitivity, specificity and accuracy of the determined cut-off and of the ≥2-fold increase cut-off. Overall, the use of pneumococcal proteins exhibited good accuracy. The use of the optimal cut-off points demonstrated a substantial increase in the accuracy for the detection of antibody responses against PcpA and a modest increase in the accuracy for the detection of responses against Ply and PspA as compared to the use of a cut-off of ≥2-fold increase in antibody levels.

We also evaluated the sensitivity and specificity of antibody responses (using the optimal cut-offs from Table 2) against different numbers of pneumococcal proteins for the diagnosis of *S. pneumoniae* infection (Table 3). The detection of antibody response against only one antigen yields good sensitivity and specificity for the diagnosis of infection by *S. pneumoniae*, when using a serological assay with all the antigens studied herein.

Finally, the effect of different levels of antibodies in the acute serum samples and of the study group on the detection of antibody responses (using the cut-offs defined in this study) was investigated using logistic regression (Table 4). There was no interference of the baseline levels of antibodies on the detection of responses for PspA, PcpA, PhtD and PcsB-N. There was no difference in the sensitivity and specificity of the assay including only the four aforementioned proteins and those of the assay with all the studied proteins (data not shown).

4. Discussion

Our observations suggest that the quantitation of specific IgG against pneumococcal proteins in paired serum samples is accurate in detecting invasive pneumococcal disease. Overall, ≥2-fold increase in antibody level is useful. However, the use of different cut-off points of antibody level increases might improve the accuracy of the test for some antigens, especially for PcpA.

The role of serology for the diagnosis of pneumococcal infection has been largely debated over the past decades (Korppi et al., 2008). *S. pneumoniae* frequently colonizes the nasopharyngeal tract in pediatric patients (Bogaert et al., 2004), and this form of contact with pneumococcus may promote an increase in the level of antigen-specific antibodies (Prevaes et al., 2012). It is possible that, if the negative controls had a new carriage of pneumococcus, a higher number of antibody responses would be detected. However, a previous study demonstrated

Table 1
Comparison of baseline antibody levels and of the frequency of ≥2-fold increases in antibody levels between the positive and negative control groups.

Protein antigen	Antibody levels in the first serum sample (U/mL)[a]		P^d	Frequency of ≥2-fold increase in antibody levels (n [%])		P^e
	Positive control group (n = 13)	Negative control group (n = 23)		Positive control group (n = 13)	Negative control group (n = 23)	
Ply	189 (53–501)	773 (251–1622)	0.005	3 (23.1%)	1 (4.3%)	0.124
CbpA	140 (10.5–880)	1505 (429–8053)	<0.001	7 (53.8%)	2 (8.7%)	0.005
PspA[b]	76 (17.5–422.5)	244 (63–859)	0.115	4 (30.8%)	0 (0%)	0.012
PcpA	1221 (76.5–3232)	2417 (1025–13,645)	0.047	7 (53.8%)	2 (8.7%)	0.005
PhtD	88 (11.5–615)	1097 (404–2973)	0.001	7 (53.8%)	2 (8.7%)	0.005
StkP-C	35 (20.5–80)	422 (132–742)	<0.001	3 (23.1%)	0 (0%)	0.040
PcsB-N	433 (69–2544.5)	1357 (295–6105)	0.081	10 (76.9%)	0 (0%)	<0.001
S. pneumoniae[c]	–	–	–	12 (92.3%)	2 (8.7%)	<0.001

[a] Median [25th–75th percentile].
[b] PspA 1 and 2 were conjugated on the same bead set.
[c] Frequency of antibody response detection to at least one pneumococcal protein.
[d] Statistical value calculated with Mann Whitney U test.
[e] Statistical value calculated with Fisher exact test.

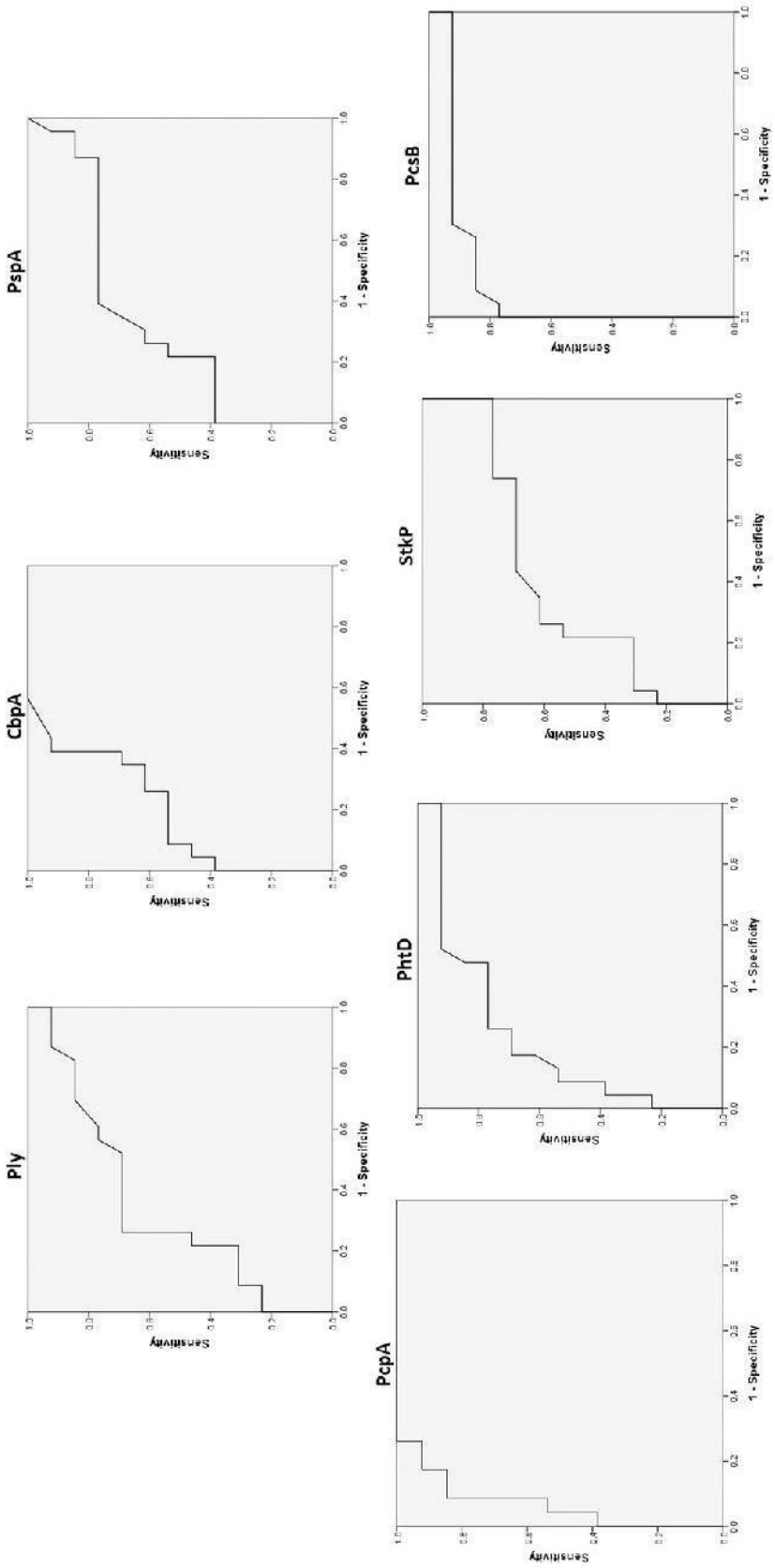

Fig. 1. Receiver operating characteristic (ROC) curve using antibody level increases for each pneumococcal antigen for the detection of pneumococcal infection in children with pneumonia. Children with invasive pneumococcal pneumonia and children with pharyngitis were used as positive and negative control groups, respectively.

Table 2
Area under the curve of the receiver operating characteristic [ROC] curves evaluating the use of fold-increases in antibody levels against each antigen for the diagnosis of pneumococcal infection, the sensitivity, specificity and accuracy of the optimal cut-off of each of these curves and of the ≥2-fold increase cut-off.

			≥Optimal cut-off[a]			≥2-Fold increase		
Antigen	Area under the curve	Optimal fold-increase	Sensitivity	Specificity	Accuracy of the cut-off	Sensitivity	Specificity	Accuracy of the cut-off
Ply	0.666	≥3.16	23.08%	100%	72.22%	23.08%	95.65%	69.44%
CbpA	0.814	≥2.85	46.15%	95.65%	77.78%	53.85%	91.30%	77.78%
PspA	0.677	≥1.97	38.46%	100%	77.78%	38.46%	95.65%	75.00%
PcpA	0.933	≥1.52	84.62%	91.30%	88.89%	53.85%	91.30%	77.78%
PhtD	0.783	≥2.04	53.85%	91.30%	77.78%	53.85%	91.30%	77.78%
StkP-C	0.609	≥1.77	30.77%	95.65%	72.22%	30.77%	95.65%	72.22%
PcsB-N	0.896	≥2.24	76.92%	100%	91.67%	76.92%	100%	91.67%

[a] Cut-offs defined in this investigation based on ROC curve.

that recent acquisition of a new pneumococcal colonization was not significantly associated with a ≥2-fold increase in the antibody levels to 27 pneumococcal proteins, including the ones studied herein (Turner and Turner, 2013). We have shown that the response against pneumococcal antigens was highly specific when comparing children with IPD and non-colonized controls. Although the requirement of paired samples presents an obstacle for the use of serology in clinical practice, it is still a viable option for etiology research purpose. Finally, serological techniques have been increasingly developed and overcame previous limitations on the number and type of antigens included in each assay (Andrade et al., 2014).

Many pneumococcal proteins have been described over the past decade, and their use in serological assays may have advantages compared to the commonly used capsular polysaccharides when serotype-specific data is not required. For instance, Olaya-Abril et al. recently described a protein array using 95 recombinantly produced pneumococcal proteins described *via* proteomics technique, representing the versatility of protein antigens for use in serodiagnostics (Olaya-Abril et al., 2015). All of the antigens included in our assay have been described as highly specific to *S. pneumoniae* and widely distributed among its strains (Tai, 2006; van der Poll and Opal, 2009; Giefing et al., 2008). Therefore, the use of pneumococcal proteins allows the identification of responses against *S. pneumoniae* regardless of the serotype of the infecting strain. Furthermore, the development of multiplex techniques has allowed the test of multiple antigens in the same assay (Andrade et al., 2014), representing a more economic option when a combination of antigens is used to improve the overall sensitivity of the test. Of note, multiplex assays including up to 64 pneumococcal antigens have been described, with good robustness and no effect from the multiplexing, representing a resourceful tool for the measurement of IgG against pneumococcal proteins (Jiménez-Munguía et al., 2015).

The differences found for the levels of antibodies in the acute phase sera may be explained by the difference in age between the study groups. The production of antibodies against protein antigens from *S. pneumoniae* starts at different ages for each specific antigen (Prevaes et al., 2012; Holmlund et al., 2009; Lebon et al., 2011; Simell et al., 2009; Hagerman et al., 2013; Holmlund et al., 2006; Rapola et al., 2000; Zhang et al., 2006) and, therefore, the age of the children directly influences the level of antibodies generated against such antigens. It is important to emphasize, however, that the antibody levels in the acute phase sera did not affect the association between the study group and the detection of an antibody response against PspA, PcpA, PhtD and PscB (Table 4). Indeed, the serological test composed only of PspA, PcpA, PhtD and PcsB had the same sensitivity and specificity as the test with all the studied proteins (data not shown). An independent association between study group and detection of antibody responses was not found for Ply, CbpA and StkP, and we hypothesize this was due to the small sample size in this study.

The optimal cut-offs of antibody increase against each antigen herein defined were based on the maximum accuracy for pneumococcal infection diagnosis. The accuracy of these cut-offs was overall similar to the use of a cut-off of ≥2-fold increase. However, the cut-off of ≥1.52-fold increase in antibody levels against PcpA demonstrated substantially higher accuracy compared to ≥2-fold increase in antibodies against the same antigen (88.89% *vs.* 77.78%). There was only a modest increase in the accuracy of the test with the use of a cut-off of ≥3.16-fold increase in antibodies against Ply and a cut-off of ≥ 1.97-fold increase in antibodies against PspA compared to the cut-off of ≥2-fold rise in antibodies against these same antigens (72.22% *vs.*69.44% and 77.78% *vs.* 75%, respectively). Therefore, the use of ≥2-fold increase in antibody levels may be a useful diagnostic criterion of pneumococcal infection when using the protein antigens investigated herein, but the use of distinct cut-offs can further improve the accuracy of the test, especially for antibody responses against PcpA. The use of a ≥2-fold increase in antibody levels had already been validated for Ply by measuring the levels of antibodies in healthy Finnish children (Nohynek et al., 1995), which contrasts with the higher optimal cut-off of ≥3.16 found herein. However, when comparing the specificity of the optimal cut-off with the accuracy of the ≥2-fold increase cut-off (100% *vs.* 95.65%, respectively), there was only a small increase in the specificity of the test by using the optimal cut-off. Therefore, the use of a ≥2-fold increase cut-off still represents a reasonable option for the diagnosis of pneumococcal pneumonia using this protein.

The use of combinations of pneumococcal protein antigens represents an interesting strategy to improve the sensitivity of a serological assay. We have shown that when using all the antigens included in this study, the detection of a response against at least one antigen is highly sensitive and specific for the presence of pneumococcal infection.

Table 3
Sensitivity, specificity and accuracy of antibody responses against different numbers of pneumococcal proteins for the diagnosis of *Streptococcus pneumoniae* infection (considering the cut-offs from Table 2 and the gold-standard of ≥2 fold increase).

Antibody responses to number of pneumococcal antigens	≥Cut-off of higher accuracy			≥2 Fold		
	Sensitivity	Specificity	Accuracy of the cut-off	Sensitivity	Specificity	Accuracy of the cut-off
≥1	92.31%	91.30%	91.67%	92.31%	91.30%	91.67%
≥2	84.62%	91.30%	88.89%	84.62%	91.30%	88.89%
≥3	69.23%	91.30%	83.33%	53.85%	91.30%	77.78%
≥4	46.15%	91.30%	75.00%	38.46%	91.30%	72.22%
≥5	30.77%	95.65%	72.22%	23.08%	95.65%	69.44%
≥6	23.08%	95.65%	69.44%	15.38%	95.65%	69.44%
≥7	15.38%	100%	69.44%	15.38%	100%	69.44%

Table 4
Effect of the study group on the frequency of antibody response detection using the cut-offs defined in this study, evaluated by logistic regression, adjusted by the antibody levels in the first serum samples.

Protein antigen	Group	
	Non adjusted odds ratio (95% CI)	Adjusted odds ratio (95% CI)
Ply		
Study group	6.6 (0.609–71.557)	1.906 (0.123–29.528)
Antibody level on the first serum sample	1.009 (0.998–1.02)	1.008 (0.996–1.02)
CbpA		
Study group	12.25 (1.996–75.196)	6.801 (0.836–44.253)
Antibody level on the first serum sample	1.001 (1–1.002)	1.0004 (0.999–1.002)
PspA[a]		
Study group	13.75 (1.386–136.387)	12.188 (1.183–125.611)
Antibody level on the first serum sample	1.002 (0.998–1.005)	1.001 (0.998–1.004)
PcpA		
Study group	57.75 (7.134–467.519)	45.566 (5.505–377.177)
Antibody level on the first serum sample	1.001 (0.999–1.0003)	1.001 (0.999–1.0003)
PhtD		
Study group	12.25 (1.996–75-196)	7.385 (1.01–54.01)
Antibody level on the first serum sample	1.001 (1–1.003)	1.0004 (0.999–1.002)
StkP-C		
Study group	9.778 (0.957–99.943)	1.937 (0.12–31.319)
Antibody level on the first serum sample	1.016 (0.993–1.039)	1.013 (0.988–1.038)
PcsB-N		
Study group	73.333 (6.764–795.076)	65.906 (5.825–745.67)
Antibody level on the first serum sample	1.001 (0.999–1.004)	1 (0.9996–1.0004)

[a] PspA 1 and 2 were conjugated on the same bead set.

It is important to highlight that, among the studied antigens, PcpA and PcsB-N have shown the highest accuracy rates, and their inclusion should be considered when developing a serological assay against *S. pneumoniae*. Indeed, Posfay-Barbe et al. showed that the use of multiple antigens markedly improves the sensitivity of antibody response detection against *S. pneumoniae*, particularly if PcpA was included in the combination (Posfay-Barbe et al., 2011). Furthermore, Jiménez-Munguía et al. also described that the levels of specific IgG against PcpA were three times lower in sera of children with pneumonia collected up to 10 days of disease, when compared to that of negative controls (Jiménez-Munguía et al., 2015). Similar results were found for the evaluation of antibody responses in a cohort of 690 children with non-severe community-acquired pneumonia, in which PcpA and PcsB were the antigens with the highest rate of antibody response detection (Borges et al., 2015).

The limitations of this study should be noted. First, there were important differences between the positive and negative controls, *i.e.*, nationality, age, vaccination status and different levels of antibodies in the first serum samples. However, we demonstrated that these differences did not affect the detection of antibody responses to at least four pneumococcal proteins, including PcpA and PcsB-N (Table 4). In addition, a recent study has demonstrated that vaccination with PCV10 did not affect the rate of antibody responders in a cohort of children with non-severe CAP (Andrade et al., in press). Second, the positive and negative control groups included in this study had few participants. It is possible that the low number of participants in this study may have compromised the independent association between the study group and the detection of antibody responses against Ply, CbpA and StkP. This is due to the difficulty in acquiring paired serum samples from pediatric patients. Furthermore, less than 5% of the pediatric cases of community-acquired pneumonia develop bacteremia (Shah et al., 2010).The negative control group was composed of children with pharyngitis, who were not strictly healthy children. However, it is important to recall that pharyngitis in children aged under 5 years is predominantly caused by viruses, and *S. pneumoniae* is not recognized as a bacterial causative agent in this setting (Hsieh et al., 2011). In fact, it is possible that the antibody responses against *S. pneumoniae* detected in the two patients with pharyngitis was caused by a polyclonal response elicited by EBV (Freijd and Rosén, 1984) or by cross reactive antibodies against Group A Streptococcus. Secondly, colonization was not evaluated at the collection of the second serum samples in the negative control group. Consequently, it is possible that the two children from the negative control group who had seroresponses against *S. pneumoniae* presented a new colonization by this bacterium between the collection of serum samples. Finally, the evaluation of colonization by *S. pneumoniae* by oropharyngeal swabs is not the most sensitive method to identify this type of contact with the pneumococcus (Satzke et al., 2013).

In conclusion, we demonstrated that serology using multiple pneumococcal proteins is a promising method for the diagnosis of pneumococcal infection in children with pneumonia. Although the cut-off of ≥2-fold increase in antibody levels is adequate for most antigens, different cut-offs may be optimal for some antigens, such as ≥1.52-fold increase in antibody levels against PcpA. Furthermore, when using the antigens studied herein, the detection of antibody response against at least one antigen is highly sensitive and specific for the diagnosis of infection by *S. pneumoniae*.

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Conflict of interests

Andreas Meinke is an employee at Valneva Austria GmbH.

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V. Artigo 2

DETERMINATION OF AVIDITY OF IGG AGAINST PROTEIN ANTIGENS FROM STREPTOCOCCUS PNEUMONIAE: ASSAY DEVELOPMENT AND PRELIMINARY APPLICATION IN CLINICAL SETTINGS

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ORIGINAL ARTICLE

Determination of avidity of IgG against protein antigens from *Streptococcus pneumoniae*: assay development and preliminary application in clinical settings

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Abstract The measurement of antibody levels is a common test for the diagnosis of *Streptococcus pneumoniae* infection in research. However, the quality of antibody response, reflected by avidity, has not been adequately evaluated. We aimed to evaluate the role of avidity of IgG against eight pneumococcal proteins in etiologic diagnosis. Eight pneumococcal proteins (Ply, CbpA, PspA1 and 2, PcpA, PhtD, StkP-C, and PcsB-N) were used to develop a multiplex bead-based avidity immunoassay. The assay was tested for effects of the chaotropic agent, multiplexing, and repeatability. The developed assay was applied to paired samples from children with or without pneumococcal disease (n = 38 for each group), determined by either serology, polymerase chain reaction (PCR), or blood culture. We found a good correlation between singleplex and multiplex assays, with $r \geq 0.94$.The assay was reproducible, with mean inter-assay variation ≤ 9% and intra-assay variation < 6%. Children with pneumococcal disease had lower median avidity indexes in the acute phase of disease for PspA1 and 2 (p = 0.042), PcpA (p = 0.002), PhtD (p = 0.014), and StkP-C (p < 0.001). When the use of IgG avidity as a diagnostic tool for pneumococcal infection was evaluated, the highest discriminative power was found for StkP-C, followed by PcpA (area under the curve [95% confidence interval, CI]: 0.868 [0.759–0.977] and 0.743 [0.607–879], respectively). The developed assay was robust and had no deleterious influence from multiplexing. Children with pneumococcal disease had lower median avidity against five pneumococcal proteins in the acute phase of disease compared to children without disease.

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Introduction

Streptococcus pneumoniae is an important cause of morbidity and mortality in children worldwide [1]. The evaluation of the antibody response against capsular polysaccharides of this bacterium has been largely used as a tool for diagnostic purposes and to evaluate response to pneumococcal vaccines [2–4]. Over the past several decades, however, several new protein antigens from *S. pneumoniae* have been identified and recognized as specific and conserved antigens [5–7], suitable for use in diagnostic assays and experimental vaccines. Promising antigens include proteins involved in the pathogenesis of pneumococcal infections and which interact in many ways with the host immune system, such as pneumolysin (Ply), choline binding protein A (CbpA), pneumococcal surface protein A (PspA), pneumococcal choline binding protein A (PcpA), pneumococcal histidine triad protein D (PhtD),

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serine/threonine protein kinase (StkP), and protein required for cell wall separation of group B streptococcus (PcsB). Ply is a highly conserved cytotoxin released during autolysis [6, 7]; CbpA and PspA are choline binding proteins which share molecular similarities [6–10]; PcpA and PhtD are surface proteins which function as adhesins [11–13]; and StkP and PcsB are immunogenic proteins recently discovered, which are supposed to play roles in cell division and peptidoglycan metabolism, respectively [14–16]. Indeed, new vaccine formulations using the aforementioned pneumococcal proteins are being tested in both human and animal trials [17–19], and serological assays using these antigens are being developed and validated [20–22].

The antibody response against pneumococcal antigens is usually evaluated through the measurement of antigen-specific antibody levels. However, samples collected at different time points are required when the quantitation of antibodies is used for diagnostic purposes, representing a limitation of this method in the clinical setting. Therefore, assays able to evaluate the effectiveness of an antibody response using only one serum sample are warranted. The functionality of antibodies against a specific antigen reflects the quality of the immune response, and may be evaluated through antibody avidity assays [2]. Avidity represents the strength of antigen–antibody binding, and has been used as a diagnostic tool for viral infections [23, 24]. Furthermore, it has been reported to increase following exposure to *S. pneumoniae* [2, 25]. To date, the avidity of IgG antibodies against pneumococcal proteins has only been evaluated in a few studies, mostly experimental vaccine trials [17, 18]. The objectives of this study were to validate a multiplex avidity assay using eight pneumococcal protein antigens, and to apply the developed assay to a clinical setting composed of children with and without pneumococcal disease with samples collected at admission and during convalescence.

Materials and methods

Reagents

A multiplexed avidity assay was designed using eight distinct recombinant pneumococcal protein antigens: Ply, CbpA, PspA family 1 (PspA1), PspA family 2 (PspA2), PcpA, PhtD, StkP-C (a C-terminal fragment of StkP), and PcsB-N (an N-terminal fragment of PcsB). A truncated PcpA [26] and PhtD [27] were supplied by Sanofi Pasteur (Sanofi Pasteur S.A., Marcy-L'Etoile, France); Ply [28], CbpA [29], and PspA1 (UAB055) [8] were supplied by Prof. Elaine Tuomanen at St. Judes Children's Research Hospital (Memphis, TN, USA); PspA 2 was supplied by Pat Coan and Profs. Susan Hollingshead and David Briles at the University of Alabama (Birmingham, AL, USA); and StkP-C and PcsB-N were supplied by Valneva Austria GmbH (Vienna, Austria) [14]. Protein antigens included in this assay were chosen based on previous data on immunogenicity profiles [6–14] and natural development of antibodies [30].

Hydroxysulfosuccinimide (Sulfo-NHS) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) were obtained from Thermo Fisher Scientific, Rockford, IL, USA. R-phycoerythrin (R-PE)-conjugated AffiniPure Goat Anti-Human IgG Fc_γ Fragment Specific was obtained from Jackson ImmunoResearch Laboratories, Inc. (Westgrove, PA, USA). Sodium thiocyanate 98% was obtained from Sigma-Aldrich (St. Louis, MO, USA). Carboxylated MicroPlex beads were obtained from Luminex Corporation (Austin, TX, USA). Fetal bovine serum was obtained from Life Technologies (Paisley, UK).

Serum samples

Patient sera that had been sent to the National Institute for Health and Welfare (Helsinki, Finland) for antibody testing were used without identification for optimization of the avidity assay, evaluation of the effect of Sodium thiocyanate on the antigen-conjugated beads, and to assess the optimal range of fluorescence readings for the determination of avidity indexes. Thirteen paired samples from Brazilian children with invasive pneumococcal disease (IPD) determined by either blood culture (9 cases) or blood polymerase chain reaction (PCR) using the *ply* primer (4 cases) were used as pneumococcal patients [20]. Paired samples from 13 healthy Finnish children who were submitted to elective tonsillectomy were used as non-pneumococcal patients [20]. Finally, the developed avidity assay was used to test paired samples from 50 children with non-bacteremic community-acquired pneumonia (CAP), which were selected based on the adequacy of the fluorescence readings for avidity testing. Out of these 50 patients, 25 had increases in anti-protein-specific IgG between acute and convalescent samples and 25 failed to respond to any of the studied pneumococcal antigens. The presence of serological response was defined as a $\geq$ 2-fold increase in the antibody levels for IgG against Ply, CbpA, PspA1 and 2, PhtD, StkP-C, or PcsB-N, or a $\geq$ 1.5-fold increase in the antibody levels for IgG against PcpA [20]. The flow chart for the patients whose samples were tested in this study is shown in Fig. 1. All the samples evaluated for avidity were from patients aged 1 to 57 months, out of which 5 patients were less than 6 months of age.

The use of the samples was approved by the Ethics Committee of the Federal University of Bahia in Brazil, the Ethics Committee of the National Institute for Health and Welfare in Finland (formerly National Public Health Institute), and the Ethics Committee of Satakunta Central Hospital, Pori, Finland.

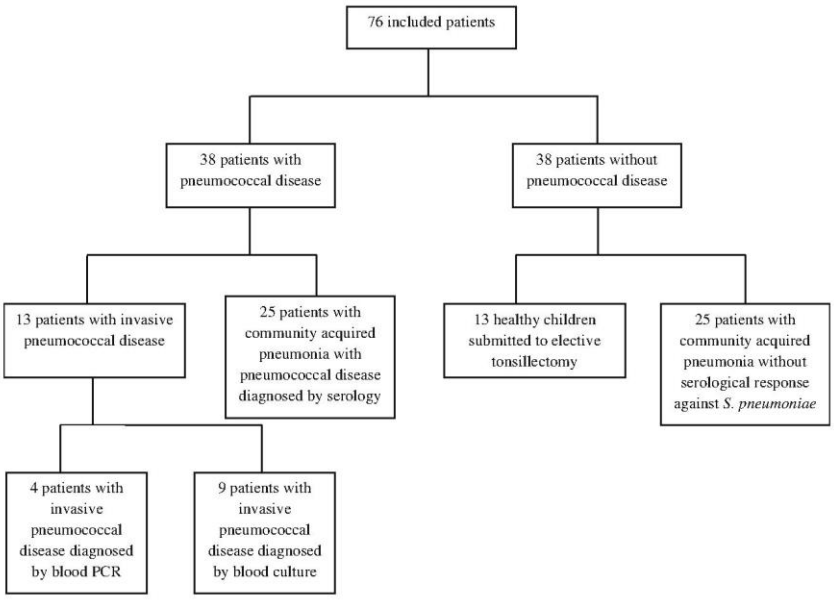

Fig. 1 Flow chart of the samples analyzed in this study

Coupling of proteins to the beads and serologic assay

A multiplexed bead-based serologic assay using Luminex xMAP® Technology to determine the levels of anti-protein-specific IgG was performed as previously described [21]. Pneumococcal proteins were coupled to activated carboxylated MicroPlex beads by a two-step carbodiimide reaction [20, 21]. Each pneumococcal protein was conjugated in one bead set, except for PspA1 and 2, which were conjugated mixed on the same bead set. Samples were analyzed using a Luminex 200 device and software (xMAP® Technology, Luminex Corporation, Austin, TX, USA).

Avidity assay

Initially, 25 μL of the F-PBS diluted serum sample in 12 replicates and 25 μL of beads diluted in F-PBS were transferred to each well of a 96-well plate (Millipore MSHVN4510, Merck KGaA, Darmstadt, Germany). Samples were diluted from 1:100 up to 1:6400, aiming to create fluorescence readings on the optimal range for the avidity assay. The plate was then incubated on a shaker at 900 rpm for 1 min and 600 rpm for 60 min at room temperature in the dark. After incubation, the plate was washed twice with PBS using a vacuum washer, and 25 μL of a solution of the chaotropic agent (Sodium thiocyanate in PBS) at six different concentrations was added: 0, 0.5, 1, 2, 4, and 6 M. The mixture was incubated for 15 min at 600 rpm in the dark and the plate was washed twice with PBS using a vacuum washer. Finally, 50 μL of a 1/100 dilution of R-PE conjugated anti-human IgG in PBS was added and the plate was incubated at 900 rpm for 1 min and 600 rpm for 30 min at room temperature. The plate was then washed as above and 80 μL/well of PBS was added. True duplicates were used throughout the development of this protocol and the median fluorescence intensity (MFI) values were averaged. The avidity index was calculated as the molar concentration of Sodium thiocyanate required to elute 50% of the bound specific antibody in a given sample [2, 3, 31].

Determination of the effect of Sodium thiocyanate on conjugated beads

Beads conjugated with each of the studied antigens were tested for the effect of a 6 M solution of Sodium thiocyanate in PBS to ensure that Sodium thiocyanate does not disrupt the antigenic structures of the antigens. A quantity of 25 μL of bead solution was incubated with either 25 μL of a 6 M Sodium thiocyanate solution or PBS for 15 min at 600 rpm in the dark. After incubation, the plate was washed twice with a vacuum washer and serial 1:4 dilutions of a serum sample with high levels of IgG against the evaluated antigens was added and the assay proceeded as described previously [21].

Determination of the optimal range of fluorescence for avidity testing

Samples with a distinct range of MFI for all the studied antigens were assayed for avidity as previously described. Seven serial 1:4 dilutions from two serum samples were assayed for avidity. The avidity index for each sample dilution was calculated and the range of MFI that yielded consistent avidity indexes was determined.

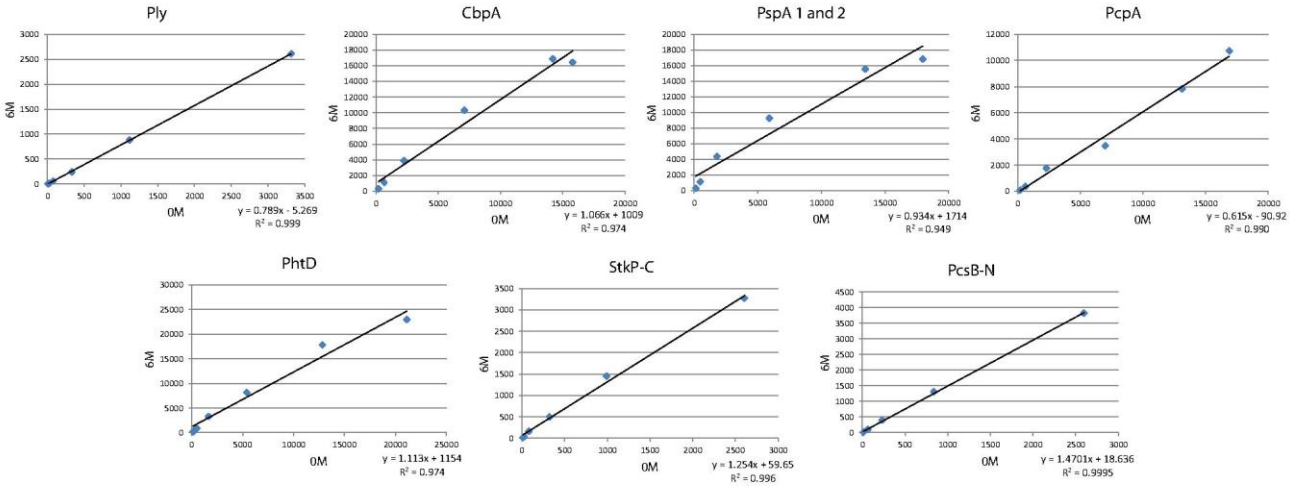

Fig. 2 Correlation between the median fluorescence intensity (MFI) values elicited by seven serial 1:4 dilutions of a serum sample with beads submitted or not to a pretreatment with 6 M Sodium thiocyanate

Comparison of singleplex and multiplex assays

The comparability between the single- and multiplex avidity assays was evaluated by determining the avidity index for a sample with high values of anti-protein IgG for all studied antigens by both formats.

Repeatability

Repeatability of the avidity assay was assessed by determining both intra- and inter-assay variation for each antigen. Intra-assay variation was calculated from eight repetitions of one sample in the same plate. Inter-assay variation was calculated from the results of two repetitions of eight samples of Brazilian children with CAP on different days. The percentage coefficient of variation (CV) was calculated for each of these results and averaged.

Evaluation of avidity in clinical settings

The avidity of IgG against pneumococcal proteins was evaluated in paired serum samples from 13 children with IPD (positive cases), 13 healthy children (negative cases), and 50 children with non-invasive CAP (25 with and 25 without serological response against *S. pneumoniae*). The avidity indexes were compared based on the group of analysis and time of sample collection (acute or convalescent samples, collected 2–4 weeks apart).

Statistical analysis

Categorical variables were presented as absolute number (percentage) and continuous variables as median (25th–75th percentiles), as they showed non-parametric distributions. Categorical variables were compared using the Chi-square or Fisher's exact test as appropriate, and continuous variables were evaluated using the Mann–Whitney *U*-test. Comparison between samples collected at different time points was performed using the Wilcoxon signed-rank test. Receiver operating characteristic (ROC) curves were plotted to determine the accuracy of avidity of IgG against pneumococcal proteins in differentiating children with or without pneumococcal disease, using either each antigen individually or combinations of different antigens. All statistical tests were two-tailed (significance level of 0.05) using SPSS software (version 9.0).

Results

Optimization of the avidity assay

Determination of the effect of Sodium thiocyanate on conjugated beads

The comparison between beads pretreated with 6 M solution of Sodium thiocyanate or PBS is shown in Fig. 2. Overall, the chaotropic agent did not have an effect on the antigens that would inhibit the binding of IgG to most conjugated beads, and similar fluorescence readings were obtained with or without treatment with Sodium thiocyanate. A decrease in the fluorescence readings was found solely for beads conjugated with PcpA. Nevertheless, we still found a good correlation between fluorescence readings obtained with or without treatment with Sodium thiocyanate for all the studied antigens, with a corresponding correlation coefficient ≥ 0.97 for all of them. Also, no difference was found when the fluorescence readings from pretreated beads with 6 M solution of Sodium thiocyanate or PBS were compared (data not shown). There was no effect from the thiocyanate treatment on background MFI levels (data not shown).

Table 1 Number of considered samples for avidity analysis, based on the presence of fluorescence readings included in the established range (median fluorescence intensity [(MFI] ranging from 100 to 7000)

	Invasive pneumococcal disease (n = 13)	Healthy children (n = 13)	Children with serological response (n = 25)	Children without serological response (n = 25)
Ply				
First serum sample	13 (100%)	12 (92%)	25 (100%)	25 (100%)
Second serum sample	12 (92%)	12 (92%)	25 (100%)	25 (100%)
Number of pairs	12 (92%)	12 (92%)	25 (100%)	25 (100%)
CbpA				
First serum sample	12 (92%)	9 (69%)	17 (68%)	16 (64%)
Second serum sample	12 (92%)	9 (69%)	19 (76%)	17 (68%)
Number of pairs	12 (92%)	8 (61%)	13 (52%)	15 (60%)
PspA1 and 2				
First serum sample	11 (85%)	10 (77%)	22 (88%)	22 (88%)
Second serum sample	11 (85%)	9 (69%)	21 (84%)	22 (88%)
Number of pairs	10 (77%)	9 (69%)	19 (76%)	20 (80%)
PcpA				
First serum sample	7 (54%)	11 (85%)	17 (68%)	20 (80%)
Second serum sample	7 (54%)	11 (85%)	10 (40%)	19 (76%)
Number of pairs	5 (38%)	10 (77%)	7 (28%)	18 (72%)
PhtD				
First serum sample	12 (92%)	12 (92%)	21 (84%)	21 (84%)
Second serum sample	13 (100%)	11 (85%)	20 (80%)	20 (80%)
Number of pairs	12 (92%)	11 (85%)	19 (76%)	19 (76%)
StkP-C				
First serum sample	3 (23%)	8 (62%)	13 (52%)	16 (64%)
Second serum sample	1 (8%)	7 (54%)	10 (40%)	18 (72%)
Number of pairs	1 (8%)	7 (54%)	7 (28%)	16 (64%)
PcsB-N				
First serum sample	13 (100%)	11 (85%)	22 (88%)	23 (92%)
Second serum sample	11 (85%)	11 (85%)	23 (92%)	23 (92%)
Number of pairs	11 (85%)	11 (85%)	21 (84%)	22 (88%)

Determination of the optimal range of fluorescence for avidity testing

The developed avidity assay gave consistent results in the MFI range from 100 up to 7000, with the coefficient of variation ranging from 1% for Ply up to 12% for PcpA. Higher values presented greater variability in the avidity index, and lower values were often out of the linear range of the serologic assay [21]. Values outside the determined linear range were excluded from the analysis. The number of samples included in the avidity analysis is shown in Table 1.

Comparison of singleplex and multiplex assays

We found good correlation between avidity indexes determined by the singleplex and multiplex assays, with a correlation coefficient ≥ 0.94 for all the studied antigens, as shown in Fig. 3. Therefore, multiplexing did not have an effect on the results of the developed avidity assay.

Repeatability

The developed avidity assay had good robustness regarding inter- and intra-assay variation. The intra-assay variation ranged from 0% for PcpA up to 6% for Ply, and the inter-assay variation ranged from 2% for CbpA up to 9% for PspA1 and 2 (data not shown).

Determination of IgG avidity in clinical settings

This study included 76 children whose median age was 19 months ([25th–75th percentile]: 12–32 months) and the median interval of serum sample collection was 21 days

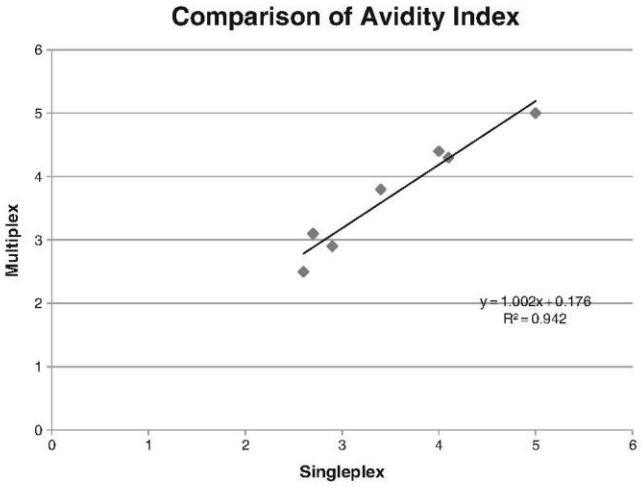

Fig. 3 Comparison of the avidity index from seven bead sets assayed in the singleplex and multiplex assays

([25th–75th percentile]: 17–32 days). Children with pneumococcal disease diagnosed by either serology, blood culture, or blood PCR had samples collected within a shorter time interval (median [25th–75th percentile]: 20 [17–25] vs. 23 [18–41] days; $p = 0.011$) when compared to the group of children without pneumococcal disease (including healthy children and children with CAP without serological response to *S. pneumoniae*), but there was no statistical difference in age (median [25th–75th percentile]: 16 [10–28] vs. 24 [14–39] months; $p = 0.054$). When evaluating solely the group of children without pneumococcal disease, children with CAP without serological response to *S. pneumoniae* were younger (median [25th–75th percentile]: 18 [12–30] vs. 29 [23–46] months; $p = 0.016$) and had samples collected at a shorter time interval (median [25th–75th percentile]: 19 [17–23] vs. 44 [40–52] days; $p < 0.001$) when compared to the subgroup of healthy children submitted to elective tonsillectomy (negative cases). There was no difference in age and sampling interval between children with IPD and children with CAP and serological response against *S. pneumoniae* (data not shown).

Initially, we compared the IgG avidity elicited in children with IPD and healthy children submitted to elective tonsillectomy, as shown in Table 2. In children with IPD, lower median IgG avidity than in healthy children was found in the acute phase for PcpA, PhtD, and PcsB-N. In the convalescent phase,

Table 2 Comparison of the avidity index obtained from children with invasive pneumococcal disease (positive cases) and healthy children submitted to elective tonsillectomy (negative cases), on the acute and convalescent serum samples

Antigen	Acute serum sample			Convalescent serum sample		
	Invasive pneumococcal disease ($n = 13$)[a]	Healthy children ($n = 13$)	p-Value	Invasive pneumococcal disease ($n = 13$)[a]	Healthy children ($n = 13$)	p-Value
Ply	4.6 (2.7–5.8)	4 (3.8–4.8)	0.810[c]	4.3 (3.2–6.5)	3.9 (3.7–4.4)	0.755[i]
CbpA	2.4 (1.9–2.7)	2.8 (2.5–3)	0.082[d]	2.5 (1.4–2.8)	2.8 (2.4–2.8)	0.277[j]
PspA1 and 2	0.8 (0.4–1.3)	1.2 (0.8–1.5)	0.173[e]	0.6 (0.4–1)	0.9 (0.7–1.6)	0.131[k]
PcpA	1.3 (0.4–1.4)	1.5 (1.4–2)	0.006[f]	1.4 (0.8–1.5)	1.5 (1.3–1.9)	0.104[l]
PhtD	2.2 (1.9–3.6)	4.2 (3.4–4.4)	0.001[g]	2.5 (1.5–3.1)	4.4 (3.6–4.5)	<0.001[m]
StkP-C[b]	–	–	–	2.2 (2.2–2.2)	3.2 (2.6–4)	0.250[n]
PcsB-N	1.8 (1.1-2.4)	3 (2.2–3.4)	0.035[h]	2.1 (1.5–2.5)	3 (2–3.3)	0.034[o]

[a] Invasive pneumococcal disease: 9 patients with positive blood culture and 4 patients with positive blood PCR

[b] Data from the first serum sample from the group with invasive pneumococcal disease was not considered for the analysis due to insufficient sampling

[c] Number of included samples: 25

[d] Number of included samples: 21

[e] Number of included samples: 21

[f] Number of included samples: 18

[g] Number of included samples: 24

[h] Number of included samples: 24

[i] Number of included samples: 24

[j] Number of included samples: 21

[k] Number of included samples: 20

[l] Number of included samples: 18

[m] Number of included samples: 24

[n] Number of included samples: 8

[o] Number of included samples: 22

Table 3 Comparison of the avidity indexes between children with pneumococcal disease diagnosed by either blood PCR and blood culture (invasive pneumococcal disease) or serology

Antigen	Acute serum sample			Convalescent serum sample		
	Invasive pneumococcal disease (n = 13)[a]	Serology (n = 25)	p-Value	Invasive pneumococcal disease (n = 13)[a]	Serology (n = 25)	p-Value
Ply	4.6 (2.7–5.8)	4.2 (3.6–5.2)	0.584[c]	4.3 (3.2–6.5)	3.9 (2.9–5.5)	0.532[i]
CbpA	2.4 (1.9–2.7)	2.6 (2.2–2.9)	0.325[d]	2.5 (1.4–2.8)	2.5 (0.9–2.8)	0.562[j]
PspA1 and 2	0.8 (0.4–1.3)	1.2 (0.6–1.9)	0.317[e]	0.6 (0.4–1)	1 (0.4–2.2)	0.271[k]
PcpA	1.3 (0.4–1.4)	1.1 (0.6–1.7)	0.455[f]	1.4 (0.8–1.5)	1.7 (1.1–2.2)	0.109[l]
PhtD	2.2 (1.9–3.6)	3 (2.4–3.8)	0.385[g]	2.5 (1.5–3.1)	2.8 (1.7–3.3)	0.456[m]
StkP-C[b]	–	–	–	2.2 (2.2–2.2)	3 (1.9–3.3)	0.545[n]
PcsB-N	1.8 (1.1-2.4)	2.5 (1.7–3.1)	0.180[h]	2.1 (1.5–2.5)	2.7 (1.8–3.1)	0.201[o]

[a] Invasive pneumococcal disease: 9 patients with positive blood culture and 4 patients with positive blood PCR

[b] Data from the first serum sample from the group with invasive pneumococcal disease was not considered for the analysis due to insufficient sampling

[c] Number of included samples: 38

[d] Number of included samples: 29

[e] Number of included samples: 33

[f] Number of included samples: 24

[g] Number of included samples: 33

[h] Number of included samples: 35

[i] Number of included samples: 37

[j] Number of included samples :31

[k] Number of included samples: 32

[l] Number of included samples: 17

[m] Number of included samples: 33

[n] Number of included samples: 11

[o] Number of included samples: 34

lower IgG avidity in the group with IPD than in healthy children was found for PhtD and PcsB-N.

Subsequently, we compared the IgG avidity indexes within the groups of children with and without pneumococcal disease. We found no difference in either acute or convalescent samples between children with pneumococcal disease diagnosed by blood PCR and blood culture or serology, as shown in Table 3. On the comparison of healthy children submitted to elective tonsillectomy and children with CAP without serological response to *S. pneumoniae*, we found that children with pneumonia had a lower median IgG avidity index against PhtD in both acute and convalescent samples. These results are shown in Table 4.

When evaluating the IgG avidity indexes from children with pneumococcal disease diagnosed by either serology, culture, or PCR and children without pneumococcal disease (including healthy children and children with CAP without serological response to *S. pneumoniae*), we found that children with pneumococcal disease had lower median IgG avidity indexes in both acute and convalescent samples, as shown in Table 5. The IgG avidity indexes for PspA1 and 2, PcpA, PhtD, and StkP-C were significantly lower in the acute sample for the group with pneumococcal disease. On the convalescent phase, significantly lower IgG avidity was found for CbpA, PspA1 and 2, PhtD, StkP-C, and PcsB-N.

There was no difference in the median IgG avidity index between acute and convalescent samples in children with and without pneumococcal disease (data not shown). A modest increase in IgG avidity between acute and convalescent samples was found among children with pneumococcal disease for PcpA (1.4 [0.9–1.8] vs. 1.5 [0.8–1.7]; p = 0.250), StkP-C (2.5 [1.3–3] vs. 3.1 [1–3.4]; p = 0.236), and PcsB-N (2.30 [1.6–3.1] vs. 2.35 [1.6–3]; p = 0.204).

Evaluation of diagnostic applications for IgG avidity

The ROC curves for PspA1 and 2, PcpA, PhtD, and StkP-C are shown in Fig. 4, as well as the number of values included in the analysis, and the accuracy, specificity, and sensitivity of the cut-offs for each antigen. The optimal cut-off points were chosen based on the highest accuracy for each antigen.

Table 4 Comparison of the avidity indexes between children without pneumococcal disease from the subgroups of healthy children submitted to elective tonsillectomy and children with community-acquired pneumonia without serological response against *S. pneumoniae*

Antigen	Acute serum sample			Convalescent serum sample		
	Healthy children (n = 13)	Children without serological response (n = 25)	p-Value	Healthy children (n = 13)	Children without serological response (n = 25)	p-Value
Ply	4 (3.8–4.8)	3.9 (3.4–4.8)	0.451[a]	3.9 (3.7–4.4)	4.4 (3.3–5.3)	0.936[h]
CbpA	2.8 (2.5–3)	2.7 (2.5–2.8)	0.388[b]	2.8 (2.4–2.8)	2.7 (2.5–2.8)	0.958[i]
PspA1 and 2	1.2 (0.8–1.5)	1.6 (1.1–2.4)	0.084[c]	0.9 (0.7–1.6)	1.4 (1–2.3)	0.086[j]
PcpA	1.5 (1.4–2)	1.6 (1.3–1.9)	1[d]	1.5 (1.3–1.9)	1.7 (1.4–2.1)	0.445[k]
PhtD	4.2 (3.4–4.4)	3.2 (2.7–3.7)	0.002[e]	4.4 (3.6–4.5)	3.2 (2.6–3.7)	0.001[l]
StkP-C	3.2 (2.8–4.3)	3.5 (3.1–4.1)	0.653[f]	3.2 (2.6–4)	3.5 (3.1–4)	0.357[m]
PcsB-N	3 (2.2–3.4)	2.7 (2–3.1)	0.445[g]	3 (2–3.3)	2.9 (2.4–3.1)	0.561[n]

[a] Number of included samples: 37
[b] Number of included samples: 25
[c] Number of included samples: 32
[d] Number of included samples: 31
[e] Number of included samples: 33
[f] Number of included samples: 24
[g] Number of included samples: 34
[h] Number of included samples: 37
[i] Number of included samples: 26
[j] Number of included samples: 31
[k] Number of included samples: 30
[l] Number of included samples: 31
[m] Number of included samples: 25
[n] Number of included samples: 34

The highest discriminative power was found for StkP-C, followed by PcpA. The area under the curve varied from 0.65 up to 0.85, and the optimal cut-offs of avidity for the diagnosis of pneumococcal disease were: < 0.9 for PspA1 and 2, < 1.2 for PcpA, < 3.1 for PhtD, and < 2.8 for StkP-C. Similar results were obtained when the ROC curves were plotted considering children with pneumococcal disease diagnosed by either serology, blood culture, or PCR as positive cases and solely the group of children with CAP without serological response against *S. pneumoniae* as negative cases (data not shown). Finally, there was an increase in the accuracy of IgG avidity for the diagnosis of pneumococcal disease by combining the avidity indexes against PcpA and StkP-C, as shown in Fig. 5, with an area under the curve of 0.871 (95% CI: 0.8–0.942). The comparison of the median difference between acute and convalescent samples for all antigens is shown in Fig. 6.

Discussion

In this report, we describe the development and validation of a multiplex avidity assay using pneumococcal proteins. When the assay was applied to a clinical setting, we found that children with pneumococcal infection present lower median avidity of IgG against protein antigens when compared to children without evidence of pneumococcal infection. Furthermore, data from the ROC curve analysis suggest that avidity studies might be useful as a diagnostic tool requiring serum sample collection only in the acute phase of disease.

The avidity of IgG against pneumococcal polysaccharides has been largely studied in vaccine trials, as a tool to evaluate the quality of the antibody response [3, 32]. Avidity tests may be used as a measure of functionality of the antibody response, and a negative correlation between antibody avidity and the antibody concentration required for opsonophagocytic/bactericidal activity has been reported in previous studies [33–35]. The use of avidity to evaluate antibody response against pneumococcal proteins, however, has only been used in a few studies, mostly experimental vaccine trials with animal models [17, 18]. Therefore, in the current setting, where new vaccines using protein antigens are under development [19], the validation of cost-effective protocols to evaluate the avidity of IgG against protein antigens is warranted. Herein, we described the validation of a robust avidity assay, in which

Table 5 Comparison of the avidity index obtained from children with and without pneumococcal disease on the acute and convalescent serum samples

Antigen	Acute serum sample			Convalescent serum sample		
	Pneumococcal disease ($n = 38$)	No pneumococcal disease ($n = 38$)	p-Value	Pneumococcal disease ($n = 38$)	No pneumococcal disease ($n = 38$)	p-Value
Ply	4.2 (3.3–5.5)	4 (3.5–4.8)	0.611[a]	3.9 (3.1–5.6)	4 (3.5–4.7)	0.965[h]
CbpA	2.6 (2.1–2.9)	2.7 (2.5–2.9)	0.150[b]	2.5 (1.2–2.8)	2.7 (2.5–2.8)	0.034[i]
PspA1 and 2	1 (0.5–1.7)	1.4 (0.9–2.1)	0.042[c]	0.9 (0.4–1.7)	1.4 (0.9–2.1)	0.026[j]
PcpA	1.2 (0.6–1.5)	1.5 (1.4–1.9)	0.002[d]	1.5 (1–1.8)	1.65 (1.4–1.9)	0.148[k]
PhtD	3 (1.9–3.7)	3.4 (2.9–4.1)	0.014[e]	2.7 (1.7–3.3)	3.6 (3.1–4.2)	< 0.001[l]
StkP-C	2.7 (1.4–3.1)	3.45 (3–4.1)	< 0.001[f]	3 (2.2–3.3)	3.5 (3–4)	0.011[m]
PcsB-N	2.2 (1.5–3.1)	2.75 (2.1–3.1)	0.072[g]	2.3 (1.6–3)	2.9 (2.4–3.2)	0.037[n]

[a] Number of included samples: 75
[b] Number of included samples: 54
[c] Number of included samples: 65
[d] Number of included samples: 55
[e] Number of included samples: 66
[f] Number of included samples: 40
[g] Number of included samples: 69
[h] Number of included samples: 74
[i] Number of included samples: 57
[j] Number of included samples: 63
[k] Number of included samples: 47
[l] Number of included samples: 64
[m] Number of included samples: 36
[n] Number of included samples: 68

there was no deleterious effect from the multiplexing or from the use of a chaotropic agent on the conjugated beads. Nevertheless, the fluorescence intensity readings (which are proportional to the IgG antibody concentrations in the sample) in this avidity assay should remain within a predetermined interval (MFI between 100 and 7000) to ensure the consistency of the results. Furthermore, multiple dilutions of the same sample may be required since the antibody concentrations to different antigens in a sample may vary.

Herein, we found that children with pneumococcal disease present lower avidity of antibodies against most evaluated protein antigens in both acute and convalescent samples, compared to children without pneumococcal disease (Table 5). Similar results were found when comparing solely children with IPD with healthy controls (Table 2). To the best of our knowledge, the avidity of IgG against pneumococcal protein antigens in clinical settings has only once been evaluated previously, in a study testing the avidity of IgG against Ply, CbpA, and PspA in a group composed of 20 children with IPD and 20 healthy controls [25]. In that study, higher avidity was found in children with IPD in the convalescent phase of disease when compared to the control group. The results from previous studies evaluating the avidity of antibodies against capsular polysaccharides, however, have also found lower avidity in children with pneumococcal disease. For instance, children with recurrent respiratory infections presented lower avidity of IgG against capsular polysaccharides when compared to healthy controls, in a study evaluating the antibody levels against 12 pneumococcal serotypes [2]. Low avidity and opsonic activity have also been reported against the infecting serotype in children with IPD [4]. In this scenario, the presence of high-avidity antibodies against pneumococcal polysaccharides has been described as a protective factor against pneumococcal infection [33, 35, 36]. It is possible, therefore, that the higher avidity against pneumococcal proteins in the group of children without pneumococcal disease found herein may also be a determinant of protection against infection by this bacterium.

Herein, we did not find a statistically significant increase in IgG avidity between acute and convalescent serum samples. An increase in avidity against pneumococcal proteins has been reported by Ota et al. [25], who found lower avidity of IgG against Ply, CbpA, and PspA in the acute phase of disease in children with IPD compared to the convalescent phase of disease. Nevertheless, these differences did not reach statistical significance. It is possible that the small sample size from

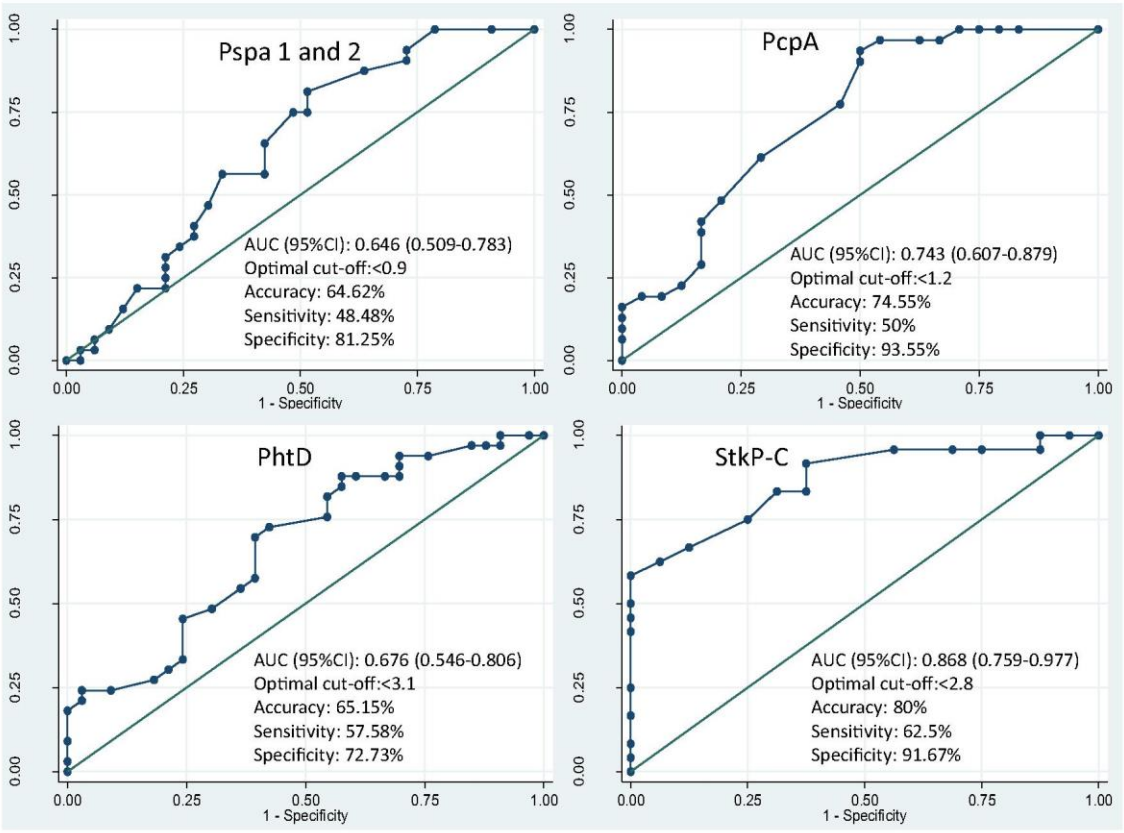

Fig. 4 Receiver operating characteristic (ROC) curves for the avidity indexes for IgG against PspA1 and 2, PcpA, PhtD, and StkP-C on the first serum sample. The number of valid values included in the analysis and optimal cut-off point for the diagnosis of pneumococcal disease with their respective accuracy, sensitivity, and specificity are shown in the graphs

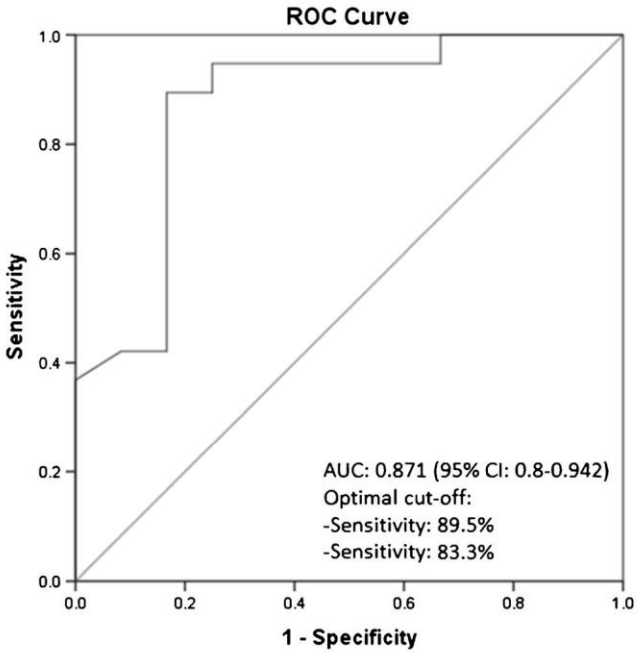

Fig. 5 Receiver operating characteristic (ROC) curves for the avidity for IgG against both PcpA and StkP-C for the first serum sample

both our study and that from Ota et al. have prevented the detection of statistically significant increases in avidity between acute and convalescent samples, which would represent the maturation of antigen-specific antibodies as a response to exposure to pneumococcal antigens.

On the evaluation of antibody avidity as a diagnostic tool for pneumococcal disease, we found that different proteins had varying discriminative powers for the detection of infection caused by *S. pneumoniae*. Overall, antibodies to StkP-C and PcpA presented high accuracy and could be considered as a candidate for an avidity assay in clinical practice. Furthermore, the use of combinations of different antigens in avidity assays may increase the accuracy of the test for the diagnosis of pneumococcal disease in children, as we showed herein for a combined assay of PcpA and StkP-C. The use of avidity has the advantage of requiring only one serum sample to provide diagnostic information, compared to serological assays, which required paired samples. For instance, the avidity of IgG against pneumococcal polysaccharides was evaluated for diagnostic purposes by Fried et al. [2], who described a high discriminative power for avidity to distinguish between groups of children with recurrent bacterial

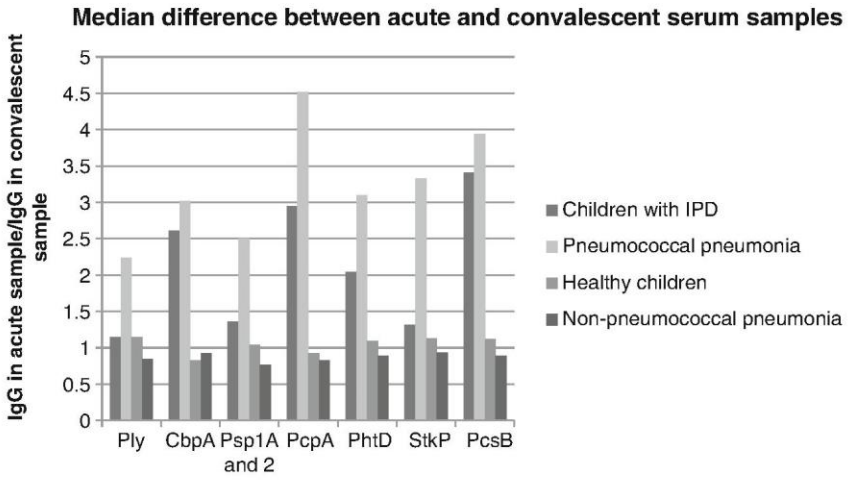

Fig. 6 Comparison of the median difference between acute and convalescent samples among children with invasive pneumococcal disease, children with pneumococcal pneumonia, healthy children, and children with non-pneumococcal pneumonia

respiratory infections and healthy controls. Nevertheless, the adequate validation of avidity protocols and definition of cut-off points for the diagnosis of acute disease is still required, particularly for assays including pneumococcal proteins that have only recently been described. Herein, we presented a preliminary evaluation of the optimal cut-offs of avidity for the diagnosis of pneumococcal disease and found a large range of avidity indexes when the cut-off points were chosen based on the highest accuracy for each protein. This finding reinforces the need for an individualized validation for each protein antigen.

The limitations of this study should be noted. Firstly, there were important differences within the group of children without pneumococcal disease (i.e., between the subgroup of healthy children and the subgroup of children with CAP without serological response to *S. pneumoniae*), such as nationality, age, and sampling interval. However, when the avidity of IgG between the subgroups was evaluated, a difference between the subgroups was found only for PhtD. It is possible that the older age in the subgroup of healthy children may have contributed to the higher avidity found against PhtD due to the longer time of possible exposition to *S. pneumoniae*. Secondly, a considerable amount of samples were excluded from the analysis due to the detection of antibody levels outside the determined optimal range. Unfortunately, repetitions could not be performed for such samples due to material and time restrictions. It is important to emphasize, however, that this was a preliminary study aiming to standardize and apply the first avidity assay in multiplex against eight pneumococcal antigens, and the described protocol should be re-evaluated using a larger panel of patient samples. Secondly, we included samples of five children aged under 6 months of age, who could still be, theoretically, under the protection of maternal antibodies. It is important to note, however, that antibody increases against pneumococcal proteins have already been reported in children aged under 6 months in the setting of symptomatic respiratory infections, such as CAP and acute otitis media [20, 37, 38]. Therefore, as young children can produce significant quantitative antibody responses despite the presence of maternal antibodies, we hypothesize that avidity should also be affected during pneumococcal infection. Thirdly, there was a decrease in the fluorescence readings for PcpA following treatment with 6 M Sodium thiocyanate. Nevertheless, we still found a high correlation between beads conjugated with this antigen which were pretreated with Sodium thiocyanate or not (Fig. 2). Finally, we had no data on colonization of the included children by *S. pneumoniae*, and how this form of contact with the pneumococcus could affect the avidity of anti-protein IgG. It has been reported that children with social mixing with other children, a risk factor to pneumococcal colonization, had higher avidity of IgG against some pneumococcal serotypes than lone children [39]. The effect of colonization with *S. pneumoniae* on the avidity of antibodies against pneumococcal proteins should be the focus of future studies.

In conclusion, this was the first report of the development and validation of an avidity assay in multiplex using pneumococcal proteins, which was robust and had no deleterious effect from multiplexing. When applied to a clinical setting, the described assay was able to identify differences in avidity of anti-protein IgG between groups of children with and without pneumococcal disease, with lower avidity found in the group of children with pneumococcal disease. The avidity of antibodies against pneumococcal proteins may also be used as a diagnostic tool for pneumococcal infection, and the protein antigens StkP-C and PcpA should be considered for inclusion in such an assay.

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Data availability statement The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The use of the samples was approved by the Ethics Committee of the Federal University of Bahia in Brazil, the Ethics Committee of the National Institute for Health and Welfare in Finland (formerly National Public Health Institute), and the Ethics Committee of Satakunta Central Hospital, Pori, Finland.

Informed consent Written informed consent was obtained from legal guardians before recruitment.

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VI. Artigo 3

INFECTION BY *STREPTOCOCCUS PNEUMONIAE* IN CHILDREN WITH OR WITHOUT RADIOLOGICALLY CONFIRMED PNEUMONIA

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ORIGINAL ARTICLE

Infection by *Streptococcus pneumoniae* in children with or without radiologically confirmed pneumonia☆

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KEYWORDS
Bacterial infection;
Etiology;
Lower respiratory tract infection;
Radiological study;
Serological tests

Abstract
Objective: Community-acquired pneumonia is an important cause of morbidity in childhood, but the detection of its causative agent remains a diagnostic challenge. The authors aimed to evaluate the role of the chest radiograph to identify cases of community-aquired pneumonia caused by typical bacteria.
Methods: The frequency of infection by *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Moraxella catarrhalis* was compared in non-hospitalized children with clinical diagnosis of community acquired pneumonia aged 2–59 months with or without radiological confirmation (n = 249 and 366, respectively). Infection by *S. pneumoniae* was diagnosed by the detection of a serological response against at least one of eight pneumococcal proteins (defined as an increase ≥2-fold in the IgG levels against Ply, CbpA, PspA1 and PspA2, PhtD, StkP-C, and PcsB-N, or an increase ≥1.5-fold against PcpA). Infection by *H. influenzae* and *M. catarrhalis* was defined as an increase ≥2-fold on the levels of microbe-specific IgG.

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Results: Children with radiologically confirmed pneumonia had higher rates of infection by *S. pneumoniae*. The presence of pneumococcal infection increased the odds of having radiologically confirmed pneumonia by 2.8 times (95% CI: 1.8–4.3). The negative predictive value of the normal chest radiograph for infection by *S. pneumoniae* was 86.3% (95% CI: 82.4–89.7%). There was no difference on the rates of infection by *H. influenzae* and *M. catarrhalis* between children with community-acquired pneumonia with and without radiological confirmation.
Conclusions: Among children with clinical diagnosis of community-acquired pneumonia submitted to chest radiograph, those with radiologically confirmed pneumonia present a higher rate of infection by *S. pneumoniae* when compared with those with a normal chest radiograph.

PALAVRAS-CHAVE
Infecção bacteriana;
Etiologia;
Infecção do trato respiratório inferior;
Estudo radiológico;
Testes sorológicos

Infecção por *Streptococcus pneumoniae* em crianças com ou sem pneumonia radiologicamente confirmada

Resumo
Objetivos: O objetivo deste estudo foi avaliar o papel do raio-X de tórax na identificação de casos de pneumonia adquirida na comunidade (PAC) causada por agentes bacterianos.
Métodos: A frequência de infecção por *Streptococcus pneumoniae*, *Haemophilus influenzae* e *Moraxella catarrhalis* em crianças com PAC não hospitalizadas foi comparada com a presença de confirmação radiológica da pneumonia (n = 249 crianças com pneumonia radiologicamente confirmada e 366 crianças com raio X de tórax normal). Infecção por *S. pneumoniae* foi diagnosticada com base na resposta sorológica a pelo menos uma dentre oito proteínas pneumocócicas investigadas (aumento ≥2 vezes nos níveis de IgG em relação a Ply, CbpA, PspA1 e 2, PhtD, StkP-C e PcsB-N ou aumento≥1,5 vezes em relação aPcpA). Infecção por *H. influenzae* e *M. catarrhalis* foi definida por aumento≥2 vezes nos níveis de IgG específica a antígenos de cada agente.
Resultados: Crianças com pneumonia radiologicamente confirmada apresentaram maior taxa de infecção pelo pneumococo. Além disso, a presença de infecção pneumocócica foi um fator preditor de pneumonia radiologicamente confirmada, aumentando sua chance de detecção em 2,8 vezes (IC 95%: 1,8-4,3). O valor preditivo negative do raio X normal para a infecção por *S. pneumoniae* foi 86,3% (IC95%: 82,4%-89,7%). Não houve diferença nas frequências de infecção por *H. influenzae* e *M. catarrhalis* entre crianças com PAC com ou sem confirmação radiológica.
Conclusões: Crianças com diagnóstico clínico de PAC submetidas a um raio X de tórax que apresentam confirmação radiológica tem maior taxa de infecção por *S. pneumoniae*, comparado às crianças com raio X normal.

Introduction

Community acquired-pneumonia (CAP) is an important cause of morbidity and mortality in childhood.[1] However, the etiologic diagnosis of CAP is challenging. Chest radiographs have been used as a diagnostic tool by the identification of radiologic patterns suggestive of an inflammatory process, such as pulmonary infiltrates. Nevertheless, the role of chest radiograph in pediatric CAP remains controversial, due to problems observed in the routine use of this exam, such as poor inter-observer concordance[2] and the inability to distinguish between distinct etiologic agents.[3,4] In turn, a significant proportion of children with a clinical diagnosis of CAP present normal chest radiograph upon admission,[5] and important differences in admission and evolution have been reported among children with CAP with or without radiological confirmation.[6–9] Altogether, these data suggest that the disease in children with or without radiologically confirmed pneumonia might be caused by distinct mechanisms and/or different etiologic agents.

In Brazil, *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Moraxella catarrhalis* have been reported as important bacterial agents of pediatric pneumonia in hospitalized children.[10] Herein, the presence of infection by *S. pneumoniae*, *H. influenzae*, and *M. catarrhalis* was investigated in non-hospitalized Brazilian children aged 2–59 months with clinical diagnosis of pneumonia with or without radiological confirmation. In doing so, the authors aimed to evaluate the role of the chest radiograph to identify probable cases of CAP caused by typical bacteria.

Methods

Study design and participants

This prospective cohort study was part of a clinical trial that evaluated the use of oral amoxicillin given thrice or twice daily to 2–59 months-old children diagnosed with CAP (PNEUMOPAC-Efficacy trial, ClinicalTrials.gov NCT01200706).[11] In that trial, 820 children were enrolled in the Emergency Department of the Universidade Federal da Bahia, in Salvador, Northeast Brazil, from November 2006 to May 2011. All children had a chest radiograph (frontal and lateral views) taken on admission, and blood samples were collected both at admission and at the follow-up visit, two to four weeks later. Inclusion criteria comprised the report of respiratory complaints and detection of lower respiratory findings, along with the presence of pulmonary infiltrate/consolidation on the chest radiograph according to the interpretation of the pediatrician on duty. Legal guardians of the included patients signed an informed consent upon enrollment.

All chest radiographs were independently read by two pediatric radiologists (CAA-N and SCA), who were blinded to the clinical data. An overall agreement of 78.7% by these two pediatric radiologists was previously demonstrated.[5] If there was no concordance on the final diagnosis of any exam, this chest radiograph was then evaluated by a third radiologist (RVB). The radiologic findings were registered according to the standardized interpretation for epidemiological studies previously published by the World Health Organization.[12] Radiologically confirmed pneumonia was defined as the presence of pulmonary infiltrate or consolidation in two independent assessments.

The use of pneumococcal conjugate vaccine-10 (PCV10) was universally implemented in Salvador, Brazil, in July 2010, for children aged <2 years.[13] Every child included in the PNEUMOPAC-efficacy trial who could have received PCV10 had the vaccine card checked personally by one of the researchers (ICB) after the trial was completed. Patients who received any dose of PCV10 and those whose vaccine status could not be identified were excluded from this analysis. Patients with severe malnutrition, defined as Z-score for weight-for-age under −3.00,[14] were also excluded. Nutritional evaluation was performed using the Anthro software. Children with lower-chest in-drawing or danger signs (inability to drink, convulsions, central cyanosis, grunting in a calm child) were excluded from the PNEUMOPAC-efficacy trial, as well as those with underlying chronic diseases.

This study was approved by the Ethics Committee of the Universidade Federal da Bahia and was conducted in accordance with the principles of the Declaration of Helsinki.

Laboratory procedures

Fluorescent multiplexed bead-based immunoassay was used to quantify the levels of antibodies against protein antigens from *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* using Luminex xMAP® technology (Luminex Corporation, TX, USA).[15] This assay included eight recombinant proteins from *S. pneumoniae* (pneumolysin [Ply], choline binding protein A [CbpA], pneumococcal surface protein A families 1 and 2 [PspA1 and PspA2], pneumococcal choline binding protein A [PcpA], pneumococcal histidine triad protein D [PhtD], serine/threonine protein kinase [StkP-C, SP1732-3], and protein required for cell wall separation of group B streptococcus [PcsB-N, SP2216-1]), three recombinant proteins from *H. influenzae* (NTHi Protein D, NTHi0371-1, and NTHi0830), and five recombinant proteins from *M. catarrhalis* (MC Omp CD, MC_RH4_2506, MC_RH4_1701, MC_RH4_3729-1, and MC_RH4_4730). Nine bead sets were created using the aforementioned proteins in the following combination: Ply, CbpA, PcpA, PhtD, StkP-C, and PcsB-N were conjugated in one bead region each; PspA1 and PspA2 were conjugated in the same bead region; and all *H. influenzae* and all *M. catarrhalis* proteins were conjugated in one bead region per bacterium.

This assay provided the mean fluorescence intensity (MFI) values for each antigen and serum evaluated. The MFI value represents an indirect measure of the IgG concentration against the studied antigens. True duplicates were used throughout the procedure and their fluorescence readings were averaged. To ensure the repeatability of the assays, high and low controls were analyzed on each plate. Furthermore, acute and convalescent samples were always analyzed on the same plate. All samples were tested using 1:400 and 1:1600 dilutions and, if necessary, further dilutions were performed. The occurrence of a serological response against *S. pneumoniae* was defined as an increase in the antibody levels ≥2-fold for IgG against Ply, CbpA, PspA1 and PspA2, PhtD, StkP-C, and PcsB-N, or an increase ≥1.5-fold for IgG against PcpA, based on the validation of a sensitive and specific serological test for the diagnosis of invasive pneumococcal disease.[16] The diagnosis of infection by *S. pneumoniae* was established by the detection of serological response against any of the evaluated antigens, based on the specificity of the assay and good correlation with ELISA.[15] The sensitivity and specificity for a serological response against each antigen were previously published.[16] The occurrence of infection by *H. Influenzae* or *M. catarrhalis* was defined as an increase in antibody levels ≥2-fold between acute and convalescent samples.[15,17] All serological tests were performed by DCA and ICB at the National Institute for Health and Welfare, in Helsinki, Finland. The frequency of these infections analyzed by age distribution, interval of sample collection, and duration of disease has been published elsewhere.[17]

Statistical analysis

Categorical variables were compared using the chi-squared or Fisher's exact tests as appropriate, and continuous variables were evaluated using Mann–Whitney's *U* test, as they presented non-parametric distribution. The negative predictive value of the normal chest radiograph for the diagnosis of infection by *S. pneumoniae* was calculated. Multivariate logistic regression was performed using the presence of radiologically confirmed pneumonia as the dependent variable and infection by *S. pneumoniae* as the independent variable. This model was adjusted by age and infection by *H. influenzae* or *M. catarrhalis*. All statistical tests were two-tailed, with a significance level of 0.05. The software Stata/SE 12.0 (StataCorp. 2011. *Stata Statistical Software:*

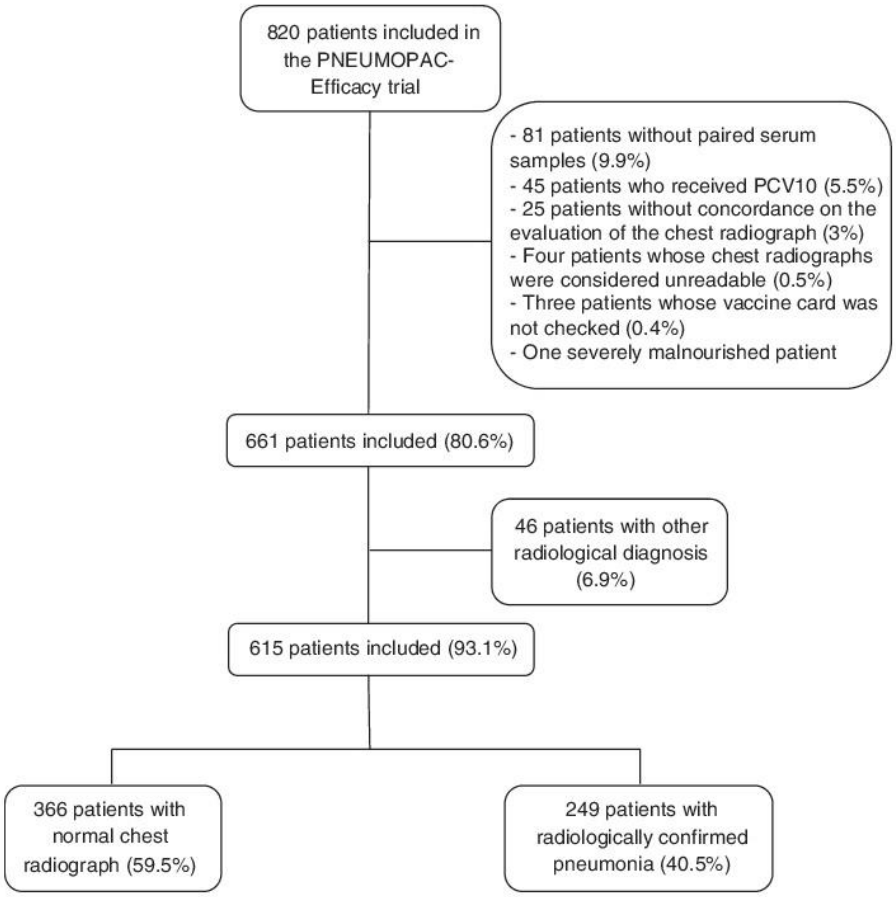

Figure 1 Flow-chart showing inclusion/exclusion criteria for children included in this study.

Release 12. College Station, TX, USA) was used to calculate the negative predictive value of the normal chest radiograph, and the software SPSS (SPSS Inc., version 9.0. Chicago, USA) was used for the remaining analyzes.

Results

Out of 820 patients included in the PNEUMOPAC-efficacy trial, 615 were included in this study, of whom 249 (40.5%) had radiologically confirmed pneumonia and 366 (59.5%) had normal chest radiograph. Fig. 1 shows the flowchart of the included and excluded cases in this investigation. Overall, 311 (50.6%) were males and the median age was 27.2 months (25th–75th percentile: 14.9–41.4 months). Consolidation was detected by radiologists 1, 2, and 3 in 84.6%, 79.8%, and 67.3% of the cases with concordant radiologically confirmed pneumonia, respectively. The remaining cases of radiologically confirmed pneumonia were diagnosed based on the detection of pulmonary infiltrates.

The comparison of the levels of antibodies on admission (first serum sample) against the studied antigens using a 1:1600 dilution factor is shown in Table 1. Children with radiologically confirmed pneumonia had significantly higher levels of antibodies against several protein antigens from *S. pneumoniae* and *H. influenzae*, and lower levels of antibodies against *M. catarrhalis* proteins. Similar results were obtained when using a 1:400 dilution factor (data not shown). Children with radiologically confirmed pneumonia also presented a higher frequency of infection by *S. pneumoniae*. Antibody responses against *S. pneumoniae* proteins were detected in 28.5% of the children with radiologically confirmed pneumonia and in 13.7% of children with a normal chest radiograph ($p < 0.001$). Antibody responses against PcpA, PhtD, and PcsB were most frequently detected in children with radiologically confirmed pneumonia. These results are shown in Table 2. When the levels of antibodies against the studied antigens on the second serum sample were compared using a 1:1600 dilution factor, higher levels of IgG against all proteins from *S. pneumoniae* and *H. influenzae* were observed, as well as lower levels of antibodies against *M. catarrhalis*, as shown in Table 3. Similar results were obtained when using a 1:400 dilution factor (data not shown).

A multivariate logistic regression was performed to assess the effect of infection by *S. pneumoniae* on the presence of radiologically confirmed pneumonia, adjusting this model by infection by *H. influenzae* or *M. catarrhalis* and age. The presence of infection by *S. pneumoniae* increased the odds of radiologically confirmed pneumonia by 2.8 (95% CI: 1.8–4.3). The presence of infection by either *H. influenzae* or *M. catarrhalis* or the age of the child did not affect

Table 1 Comparison of the median fluorescence intensity (MFI) values from the first serum sample from children with radiologically confirmed pneumonia or those with a normal chest radiograph, using a 1:1600 dilution factor.

	Radiologically confirmed pneumonia[a] $n=249$	Normal chest radiograph[a] $n=366$	p^b
Ply	152 (69–277.5)	119.5 (60.8–231)	0.023
CbpA	5623 (1422.5–9903.5)	3316 (650–9540.8)	0.010
PspA	318 (119.5–933.5)	278.5 (88.8–809.8)	0.113
PcpA	1077 (303–1941.5)	713.5 (152.8–1643)	0.004
PhtD	2244 (547–4471.5)	1447 (404.5–3353)	0.015
StkB	299 (98–865.5)	274 (82–663)	0.116
PcsB	2215 (478.5–5546)	1682 (349.75–4064)	0.016
H. influenzae	152 (90–273.5)	128 (87–219.3)	0.047
M. catarrhalis	104 (69–153.5)	114 (81–182)	0.002

[a] Results area presented as median (interquartile range).
[b] Data evaluated by Mann–Whitney's *U* test.

Table 2 Comparison of the frequencies of antibody response against protein antigens for children with a clinical diagnosis of CAP and either radiologically confirmed pneumonia or a normal chest radiograph.

	Radiologically confirmed pneumonia $n=249$	Normal chest radiograph $n=366$	p^b
Ply	14 (5.6%)	9 (2.5%)	0.042
CbpA	19 (7.6%)	15 (4.1%)	0.060
PspA	13 (5.2%)	14 (3.8%)	0.407
PcpA	54 (21.7%)	32 (8.7%)	<0.001
PhtD	24 (9.6%)	7 (1.9%)	<0.001
StkB	21 (8.4%)	9 (2.5%)	0.001
PcsB	36 (14.5%)	7 (1.9%)	<0.001
S. pneumoniae[a]	71 (28.5%)	50 (13.7%)	<0.001
H. influenzae	18 (7.2%)	19 (5.2%)	0.297
M. catarrhalis	4 (1.6%)	9 (2.5%)	0.471

[a] Antibody response against at least one pneumococcal protein.
[b] Data evaluated using chi-square for Fisher's exact test as appropriate.

Table 3 Comparison of the median fluorescence intensity (MFI) values from the second serum sample from children with radiologically confirmed pneumonia or those with a normal chest radiograph, using a 1:1600 dilution factor.

	Radiologically confirmed pneumonia[a] $n=249$	Normal chest radiograph[a] $n=366$	p^b
Ply	147 (70–257.5)	118 (58.8–226.8)	0.009
CbpA	6226 (1514.5–10656)	3288.5 (616.8–8978.3)	0.001
PspA	309 (112–909.5)	243 (93.8–670.5)	0.049
PcpA	1149 (418–2240.5)	720.5 (150.3–1654)	<0.001
PhtD	2241 (645–5094.5)	1486.5 (401.3–3278)	0.001
StkB	333 (105.5–856)	253.5 (83.8–679.3)	0.034
PcsB	2875 (682.5–6051.5)	1632 (318.8–4304.5)	<0.001
H. influenzae	160 (94–306)	139 (92.8–221)	0.028
M. catarrhalis	95 (69–136.5)	109.5 (79–166.5)	0.001

[a] Results area presented as median (interquartile range).
[b] Data evaluated by Mann–Whitney *U*'s test.

the odds for detection of radiologically confirmed pneumonia (odds radio [95% CI]: 1.42 [0.7–2.9]; 0.4 [0.1–1.6]; and 0.9 [0.9–1], respectively). Furthermore, the negative predictive value of the normal chest radiograph for the diagnosis of infection by the pneumococcus was 86.3% (95% CI: 82.4–89.7%).

Discussion

This study demonstrated that children with radiologically confirmed pneumonia have a higher frequency of infection by *S. pneumoniae* than those with a normal chest radiograph. The presence of infection by pneumococcus was

independently associated to radiologically confirmed pneumonia among non-hospitalized children with clinical CAP. Furthermore, the presence of a normal chest radiograph had a high negative predictive value for the detection of antibody responses against *S. pneumoniae*.

A higher frequency of antibody response against several antigens from *S. pneumoniae* was observed in the group of children with radiologically confirmed pneumonia when compared with those with a normal chest radiograph. This finding corroborates the results from previous studies, which demonstrated that the presence of alveolar infiltrates on chest radiographs was associated with bacterial pneumonia.[18] For instance, Nascimento-Carvalho et al. also reported that infection by *S. pneumoniae* was more frequently detected among hospitalized children with CAP who presented radiographic pneumonia rather than those with a normal chest radiograph.[19] In turn, children with a normal chest radiograph had a higher incidence of viral infection.[19] This is the first report of the association between pneumococcal infection and radiologically confirmed pneumoniae among non-hospitalized children with clinical CAP.

Accordingly, the negative predictive value of the normal chest radiograph for the detection of pneumococcal infection was high (86.3% [95% CI: 82.4–89.7%]). Although an association between bacterial infection and alveolar infiltrates/consolidation has been previously described,[18] these findings cannot reliably establish the etiologic diagnosis of CAP.[4,5] Therefore, the present finding that the normal chest radiograph has a high negative predictive value for pneumococcal infection may aid in the interpretation of this exam. The high negative predictive value observed for the normal chest radiograph in a population with high prevalence of pneumococcal infection is noteworthy,[10] thereby reinforcing the present results. Altogether, the present data indicate that children with non-severe CAP with radiologically confirmed pneumonia have a higher chance of infection by *S. pneumoniae*, whereas children with a normal chest radiograph are not likely to present infection by this agent and might not benefit from empiric antibiotic therapy.

Data from vaccine trials reinforce the relationship between pneumococcal infection and radiologically confirmed pneumonia, as a differential effect of pneumococcal vaccination was found on the rates of pediatric CAP depending on the applied diagnostic criteria. For instance, the efficacy of the PCV10 was significantly higher for children with consolidation on the chest radiograph than for children either with alveolar infiltrates or solely with a clinical diagnosis of CAP.[20] Therefore, the greater impact of pneumococcal vaccination on children with consolidation on chest radiographs suggests that patients with this radiological diagnosis present a higher incidence of pneumococcal infection. These findings are consistent with those reported by Lucero et al., who demonstrated a good vaccine efficacy of PCV11 on children with radiographic pneumonia defined as consolidation and a practicably negligible vaccine efficacy for children with a clinical diagnosis of pneumonia.[21] These vaccine trials provide indirect evidence regarding the etiology of pneumonia in children with distinct radiological patterns, indicating that children with radiologically confirmed pneumonia indeed present a higher frequency of infection by *S. pneumoniae*.

The role of the chest radiograph in the management of children with CAP, however, has been largely debated. Importantly, Bradley et al. recommend that the chest radiograph should only be used in children who are hospitalized or with hypoxemia, significant respiratory distress, suspected complications, or therapy failure.[22] This position is corroborated by Harris et al., who stated that children with signs and symptoms suggesting pneumonia who are not admitted to hospital should not routinely have a chest radiograph.[23] These recommendations are partly due to previous studies that have shown that bacterial pneumonia cannot be differentiated from non-bacterial pneumonia based solely on the findings of an abnormal chest radiograph.[3,4,24] Furthermore, the current evidence suggests that the use of a chest radiograph does not improve the outcome of pediatric patients with CAP.[25] Nonetheless, it is important to emphasize that when the impact of the chest radiograph on the management of children with CAP was evaluated, the patients received antibiotics at the discretion of the attending physician, regardless of the radiologic findings, thereby limiting the potential benefit of a radiological study in these patients as a diagnostic tool with therapeutic implications.[25] Accordingly, Harris et al. recommend the use of antibiotics for all children with a clear diagnosis of CAP.[23] Both guidelines agree, however, that young children do not require routine use of antibiotics, as most present viral acute lower respiratory infection.[22,23] In this scenario, although the chest radiograph does not unequivocally distinguish etiologic agents of CAP, it may help differentiating distinct patterns of lower respiratory infections. Recent evidence has demonstrated important differences between children with or without radiologically confirmed pneumonia in the clinical presentation and evolution. Children with radiologically confirmed pneumonia have a higher frequency and longer persistence of fever,[6–8] and also evolve more severely, with longer hospitalization, higher need of respiratory support, and higher rates of treatment failure.[9] These differences indicate that children with and without radiologically confirmed pneumonia may have different patterns of lower respiratory tract infection, and the chest radiograph, when performed, may aid the management of doubtful cases of non-severe CAP.

It was also observed that children with radiologically confirmed pneumonia had higher levels of antibodies against several pneumococcal proteins both at admission and in convalescence. Lower levels of anti-pneumococcal antibodies on admission have been associated with a higher frequency of antibody responses against *S. pneumoniae* due to particularities of the serological methods.[17] Therefore, the level of antibodies at admission probably was not responsible for the higher rate of antibody responses against the pneumococcus in children with radiological pneumonia. The higher level of antibodies at admission in this group of children, in turn, might have been caused by previous colonization by *S. pneumoniae*. Nasopharyngeal colonization has been recognized as part of the natural history of pneumococcal disease, which ensues if immunological barriers are crossed by the colonizing bacteria.[26] Also, children with clinical and radiological pneumonia are also more frequently colonized with *S. pneumoniae* when compared with healthy controls.[27] Therefore, it is possible that a higher rate of carriage of *S. pneumoniae* in children with

radiologically confirmed pneumonia elicited the higher levels of anti-pneumococcal antibodies found in this subgroup.

No difference was observed on the rates of antibody response against *H. influenzae* and *M. catarrhalis* in this study, possibly due to the low numbers of responders within the study group. However, discretely higher levels of antibodies against *H. influenzae* were found in children with radiologically confirmed pneumonia, as well as lower levels of antibodies against *M. catarrhalis*. It is known that several bacterial agents compete to colonize the nasopharyngeal tract of pediatric patients, creating a dynamic process of turnover of colonizing agents.[27] Increased rates of colonization by *S. pneumoniae* might also have contributed to lower the levels of antibodies against *M. catarrhalis* on the samples collected from children with radiologically confirmed pneumonia at admission. In turn, a positive correlation between colonization by *S. pneumoniae* and *H. influenzae* has already been described, which may have contributed to the high levels of antibodies at admission found against *H. influenzae*.[28]

The limitations of the present study must be emphasized. Firstly, data on the colonization status of the evaluated children were not available, and the putative effect of pneumococcal carriage on the antibody levels at admission was not evaluated. Secondly, the study was composed of unvaccinated children, which does not represent the reality in most countries in the post-PCV era. Nevertheless, recent evidence suggests that the use of PCV does not interfere with the result of protein-based serological assays in children with CAP,[29] which favors the generalization of the present results. Also, data on the use of other vaccines that could have influenced the results presented herein, such as the *H. influenzae* type b vaccine, was not available. However, the coverage of the *H. influenzae* type b vaccine among the pediatric population in Brazil is high (>80%), so differential rates of vaccination probably did not affect the present results.[30] Finally, as all antigens from *H. influenzae* and *M. catarrhalis* were conjugated in one bead region per bacterium, individual fluorescence readings were not obtained for these antigens.

In conclusion, this study demonstrated that, among non-hospitalized children with clinical CAP who were submitted to a chest radiograph, those with radiologically confirmed pneumonia had a higher frequency of infection by *S. pneumoniae* compared to children with a normal chest radiograph. Furthermore, the presence of pneumococcal infection was independently associated with radiologically confirmed pneumonia; normal chest radiograph has a high negative predictive value for pneumococcal infection.

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Conflicts of interest

Andreas Meinke is an employee of Valneva Austria GmbH. The others authors declare no conflicts of interest.

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VII. Artigo 4

10-VALENT PNEUMOCOCCAL CONJUGATE VACCINE (PCV10) DECREASES METABOLIC ACTIVITY BUT NOT NASOPHARYNGEAL CARRIAGE OF *STREPTOCOCCUS PNEUMONIAE* AND *HAEMOPHILUS INFLUENZAE*

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10-valent pneumococcal conjugate vaccine (PCV10) decreases metabolic activity but not nasopharyngeal carriage of *Streptococcus pneumoniae* and *Haemophilus influenzae*

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ABSTRACT

Background: The effect of pneumococcal vaccination is widely variable when measured by nasopharyngeal carriage of vaccine and non-vaccine targets. The aim of this study was to compare the carriage rates and metabolic activity of *Streptococcus pneumoniae, Staphylococcus aureus, Haemophilus influenzae* and *Moraxella catarrhalis* among children who were or were not vaccinated with PCV10.
Methods: We included children with acute respiratory infection aged 6–23 months from a cross-sectional study (CHIADO-IVAS). Nasopharyngeal aspirates were collected and respiratory pathogens were quantified by nCounter digital transcriptomics (Nanostring) and metagenomic sequencing of 16S ribosomal RNA (Illumina). The metabolic rate was calculated by the ratio between RNA transcripts and 16S DNA reads.
Results: Out of the 80 patients in this study, 53 were vaccinated with PCV10 and 27 were unvaccinated. There was no difference in nasopharyngeal carriage rates of *S. pneumoniae, S. aureus, H. influenzae* or *M. catarrhalis* by either transcriptomic analysis or 16S metagenomics. However, unvaccinated children presented a higher metabolic rate for *S. pneumoniae* compared to PCV10-vaccinated children (Median [25–75th percentiles]: 126 [22.75–218.41] *vs.* 0[0–47.83], $p = 0.004$). Furthermore, unvaccinated children presented a positive correlation between mRNA counts and 16S DNA reads for *S. pneumoniae* ($r = 0.707$; $p < 0.001$) and *H. influenzae* ($r = 0.525$; $p = 0.005$), in contrast to vaccinated children. No such effect was observed for *S. aureus* and *M. catarrhalis*.
Conclusions: Vaccination by PCV10 exerts a pathogen-specific effect on pneumococcal metabolic rate. Pathogen RNA/DNA ratio might represent a more sensitive readout for vaccine follow-up, as compared to nasopharyngeal carriage.

1. Introduction

Nasopharyngeal colonization is a dynamic process in which commensal and potentially pathogenic bacterial agents are constantly acquired and eliminated in the nasopharynx. It represents a fundamental step for invasive disease [1–3], and affects clinical characteristics in children with viral acute respiratory infection (ARI) [4,5]. *Streptococcus pneumoniae, Haemophilus influenzae, Moraxella catarrhalis,* and *Staphylococcus aureus* are commonly carried bacteria in the nasopharynx of children, and present a complex net of interactions among them [6,7]. The use of pneumococcal conjugate vaccines (PCV) alters the dynamics of nasopharyngeal colonization by inducing pathogen-specific immune responses that change carriage rates of the targeted bacteria [8–10] and bystander effects on bacterial pathogens not targeted by the vaccine. However, the effect of pneumococcal vaccination on the metabolic profile of colonizing bacteria has not been established so far.

Transcriptomic profiling of nasopharyngeal samples is a high-throughput method to acquire dynamic phenotypic information

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from colonizing pathogens [11], and represents a tool to assess pathogen metabolic activity through the production of mRNA. It presents high accuracy when assessing pathogen replication or clearance [12,13]. The aim of this study was to compare the carriage rates and metabolic activity of *S. pneumoniae, S. aureus, H. influenzae,* and *M. catarrhalis* among children who were or were not vaccinated with PCV10.

2. Materials and methods

2.1. Patients and samples

We included patients from a prospective cross-sectional study (CHIADO-IVAS) which recruited children aged from 6 to 23 months with ARI seen at the Pediatric Emergency Department of the Federal University of Bahia Hospital, in Salvador, Northeast Brazil, between September 2009 and October 2013 [14]. Inclusion criteria for the CHIADO-IVAS study comprised the presence of either fever, sneeze, running nose, nasal blockage, or cough for up to 7 days. Patients were selected for this study based on the availability of vaccination data and nasopharyngeal aspirate (NPA) for colonization testing. In Salvador, Brazil, the use of PCV10 (Synflorix, GlaxoSmithKline Biologicals, Rixensart, Belgium) was universally introduced in July2010 for children aged <2 years. It was composed by capsular polysaccharide serotypes 1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F, and 23F conjugated to non-typable *H. influenzae* (NTHi) protein D, tetanus toxoid, or diphtheria toxoid. Clinical data was recorded in a standardized form and the vaccine card was checked for PCV application and adherence to the recommended vaccine posology. All included children received the *Haemophilus influenzae* type b (Hib) vaccine. Written informed consent was obtained from legal guardians before recruitment and the study was approved by the Ethics Committee of the Federal University of Bahia.

NPA samples were collected from included children using the following protocol: the distance between the entrance of the nostril and the ear lobe was measured as an estimate of the distance from the entrance of the nostril to the nasopharynx; an aseptic plastic sputum catheter was inserted into the nostril until reaching the nasopharynx; negative pressure was applied and approximately 2 mL of nasal secretions were collected and deposited in a sterile tube with 1 mL of Nuclisens Lysis Buffer (Biomerieux, Boxtel, The Netherlands).

2.2. Laboratory procedures

The presence of colonizing bacteria in the nasopharynx of recruited children was quantified using nCounter transcriptomic analysis (which may indicate cellular activity through RNA transcription) and metagenomic sequencing of 16S ribosomal RNA using Illumina Technology.

2.2.1. nCounter analysis

Total RNA (10–50 ng) was extracted using RNEasy (Qiagen, Hilden, Germany) and was subsequently hybridized against probes targeting *S. pneumoniae, H. influenzae, M. catarrhalis,* and *S. aureus*, using the nCounter gene expression system (Nanostring Technologies), which captures and digitally counts individual mRNA transcripts. Probes were chosen based on specificity profiles previously described in the literature [15,16]. The laboratory tests were performed at the Laboratory for Clinical and Epidemiological Virology, Rega Institute for Medical Research, KU, in Leuven, Belgium. Raw data were processed using nSolver 2.0 software (Nanostring Technologies) sequentially correcting three factors: technical variation between batches (positive control RNA), background correction (negative control) and RNA content by adjusting the counts geometric mean for the 15 housekeeping genes, followed by normalization using logarithmic transformation (base 2).

2.2.2. Metagenomic sequencing

We performed metagenomic sequencing of prokaryotic 16S ribosomal RNA gene (16S rRNA) using the standard Illumina protocol. 16S rRNA is approximately 1500 bp long and contains nine variable regions interspersed between conserved regions, which are frequently used in phylogenetic classification. Herein, we included a dual barcoding strategy to multiplex up to 96 samples per run. The V3 and V4 regions of the rRNA 16S gene were amplified and Illumina sequencing adapters and dual-index barcodes to the amplicon target were added using limited cycle PCR. The amplified DNA was sequenced with MiSeq v3 reagents using paired 300-bp reads, in which ends of each read were overlapped to generate high-quality, full-length reads of the V3 and V4 regions. Data was analyzed using a two-step strategy to maximize identification of clinically relevant bacteria at the species level. First, by using Kraken software and Krona chart visualization (on BaseSpace, Illumina), which allows a rapid and convenient overview of the complete microbiome, as well as unclassified reads as a quality control (see example in Supplementary Fig. 1). Second, after microbiome determination using Greengenes database, bacterial species level assignments (including all clinically relevant) were confirmed by QIIME [17], performing usearch, with chimera checking and quality filtering, plus blast searching in the SILVA 123 release, with 98% similarity and default e-value. Finally, we performed a correlation analysis between both fast (Kraken) and stringent (QIIME) analysis of DNA reads for both *Streptococcus pneumoniae* and *Staphylococcus aureus* (Fig. 1) and closely related species (*Streptococcus equinus and Staphylococcus aureus,* respectively), and demonstrated that the two types of analysis were concordant and highly specific.

2.3. Statistical analysis

Categorical variables were compared using chi-square or Fisher's exact test as appropriate and continuous variables were evaluated using Mann-Whitney U as they presented non-parametric distribution. Kruskal-Wallis test was used when the association of a categorical variable with more than 2 levels and a continuous variable was evaluated. Carriage of each bacterium was defined as the detection of 16S DNA in NPA, and bacterial load as the number of reads of 16S rRNA gene. The ratio between mRNA transcripts and 16SDNA reads of each bacterium was calculated to assess metabolic activity of the respective bacterium (*S. pneumoniae, S. aureus, H. influenzae,* or *M. catarrhalis).* Logistic regression was performed to assess the effect of the number of doses of PCV10 on the detection of RNA transcripts or bacterial carriage (16S DNA). Finally, results were clustered according to Spearman's correlation and shown as heatmap to assess the interplay between carried bacteria following pneumococcal vaccination. All statistical tests were two-tailed, (significance level of 0.05) using SPSS software (version 9.0).

3. Results

We included 80 patients with ARI and simultaneous quantification of both RNA transcripts and 16S DNA reads in their NPA. The flow-chart of the included and excluded patients is shown in Fig. 2. The median age was 10.1 months (25–75% percentiles: 8.1–14.7 months) and 31patients were males (38.8%). Fifty-three patients had received PCV10 (66.3%), out of which 9 (17%) had received 1 dose, 23 (43.4%) had received 2 doses, 14 (26.4%) had received 3 doses and 7 (13.2%) had received 4 doses of PCV10. Overall, carriage of *S. pneumoniae, S. aureus, H. influenzae* and

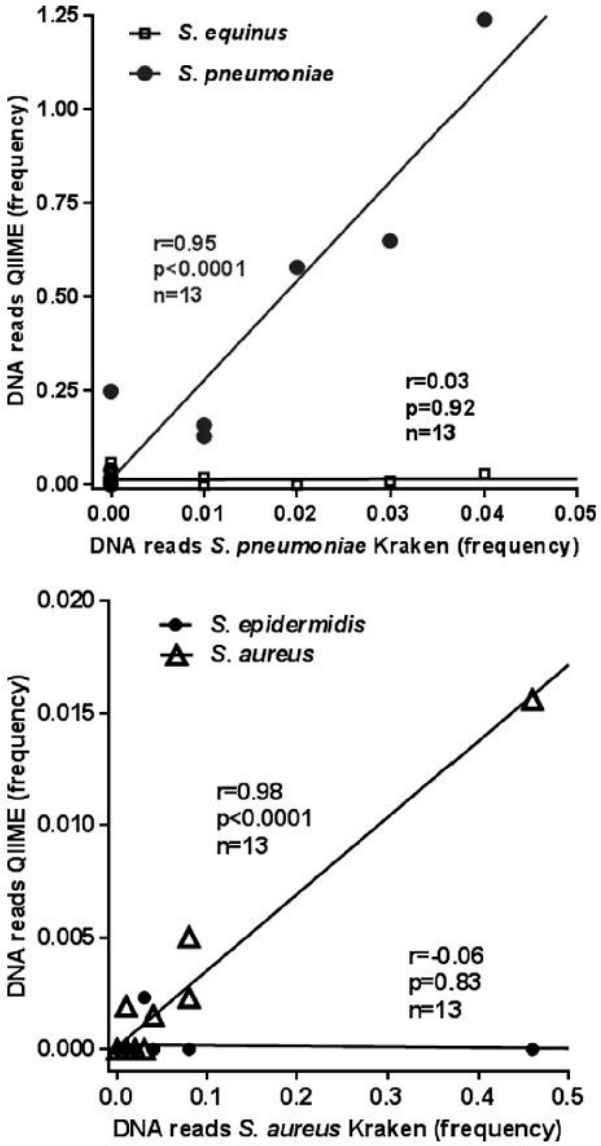

Fig. 1. Specificity of 16S metagenomics reads mapping at species level. A) Frequency of *Streptococcus pneumoniae* reads using either a fast (Kraken) or stringent protocol (QIIME) is highly concordant (Linear regression r = 0.95, p < 0.0001) and specific (no correlation to closely related *Streptococcus equinus*, r = 0.03, p = 0.92) B. Frequency of *Staphylococcus aureus* reads using either a fast (Kraken) or stringent protocol (QIIME) is highly concordant (Linear regression r = 0.98, p < 0.0001) and specific (no correlation to closely related *Staphylococcus epidermidis*, r = −0.06, p = 0.83).

M. catarrhalis was found in 38 (47.5%), 62 (77.5%), 80 (100%) and 80 (100%) patients, respectively, as defined by 16Smetagenomic analysis. All children presented carriage of bacteria from the *Streptococcus* genus. RNA transcripts of *S. pneumoniae, S. aureus, H. influenzae* and *M. catarrhalis* were detected in 27 (33.8%), 80 (100%), 77 (96.3%), 71 (88.8%) of patients, respectively.

We found no difference in detection rates of RNA transcripts or bacterial carriage (16S DNA) between children who had or had not received PCV10 (Table 1). There was also no difference in the median RNA counts or bacterial load (16SDNA reads) from *S. pneumoniae, H. influenzae* and *M. catarrhalis* between vaccinated and unvaccinated patients (Table 2). Children who received PCV10 had higher *S. aureus* RNA counts compared to unvaccinated children, but there was no difference in the number of 16S DNA reads. Moreover, there was no difference in bacterial load of the complete *Streptococcus* genus (Median [25–75th percentiles]: 14.75 [4.21–39.54] *vs.* 17.21 [2.58–39.34], p: 0.895).

The comparison of the ratio of RNA transcripts and 16S DNA reads between PCV10-vaccinated and unvaccinated children are shown in Table 3. Unvaccinated children had higher RNA/DNA ratio from *S. pneumoniae* compared to vaccinated children, reflecting a higher metabolic rate. There was no difference on the RNA/DNA ratio between vaccinated and unvaccinated children for the remaining bacteria.

Finally, we found that unvaccinated children presented a positive correlation between mRNA counts and 16S DNA reads for *S. pneumoniae* and *H. influenzae*, as shown in Fig. 3. Vaccinated children presented no correlation between mRNA transcripts and 16S DNA reads for any of the studied bacteria.

When the effect of the number of doses of PCV10 on the RNA counts or 16S DNA reads was assessed, we found differences in the RNA counts from *M. catarrhalis*(Median [25–75th percentiles]: 4.88 [1.16–5.87] *vs.* 5.75 [4.65–6.9] *vs.* 5.94 [4.87–7.38] *vs.* 3.8 [0–5.52] for children who received 1, 2 3, or 4 doses of PCV10, respectively; p = 0.039). There was no significant difference in RNA counts, 16S DNA reads or in the ratio between RNA counts and 16S DNA reads according to dosage of PCV10 for any of the remaining pathogens (data not shown). Also, there was no effect of the number of doses of PCV10 on the rates of detection of RNA transcripts or bacterial carriage (16S DNA) (data not shown).

Finally, clustering of pathogen mRNA counts and 16S DNA reads and PCV10 vaccination status (according to Spearman's correlation) revealed three independent clusters, as shown in Fig. 4. A first cluster was formed by *S. pneumoniae* 16S DNA reads, highly correlated to total *Streptococcus* genus DNA frequency. The second cluster displayed strongly correlated 16S DNA reads and mRNA counts of both *H. influenzae* and *S. aureus*. The third cluster shows strong correlation between *S. pneumoniae* replication (mRNA counts), vaccination status and *M.* RNA counts and 16S DNA reads, in agreement with the effects observed in our univariate results. Thus, PCV10 vaccination strongly influences the dynamics of both vaccine (*S pneumoniae*) and non-vaccine targets (the entire *Streptococcus* genus and *M. catarrhalis*).

4. Discussion

We found that PCV10 vaccination did not alter carriage rates by *S. pneumoniae, H. influenzae, M. catarrhalis*, and *S. aureus*. Nevertheless, colonizing pneumococci in unvaccinated children had a higher metabolic rate than in vaccinated children. Moreover, a significant correlation between RNA transcripts and 16S rRNA DNA reads was found only for *S. pneumoniae* and *H. influenzae* in unvaccinated children. Altogether, these findings indicate that pneumococcal conjugate vaccination affects the biological activity of bacterial respiratory pathogens in the nasopharynx of children.

Pneumococcal vaccination promotes a decrease in vaccine-covered *S. pneumoniae* serotypes associated with an increase in non-vaccine-covered serotypes [8,18], which has been reported to result in an unaltered overall frequency of pneumococcal carriage [19]. Also, recent evidence suggests that PCV10 does not reduce the carriage rates by *H. influenzae* despite the presence of NTHi protein D in its composition [8,18]. No difference in carriage rates of *M. catarrhalis* or *S. aureus* have been reported following pneumococcal vaccination in a clinical trial evaluating the effectiveness of PCV10 in nasopharyngeal colonization of pathogenic bacteria [8]. Accordingly, we found no difference on the carriage rates of either *S. pneumoniae, H. influenzae, M. catarrhalis* or *S. aureus* between PCV10 vaccinated or unvaccinated children. However, PCV use also induces changes in the metabolic profile of colonizing

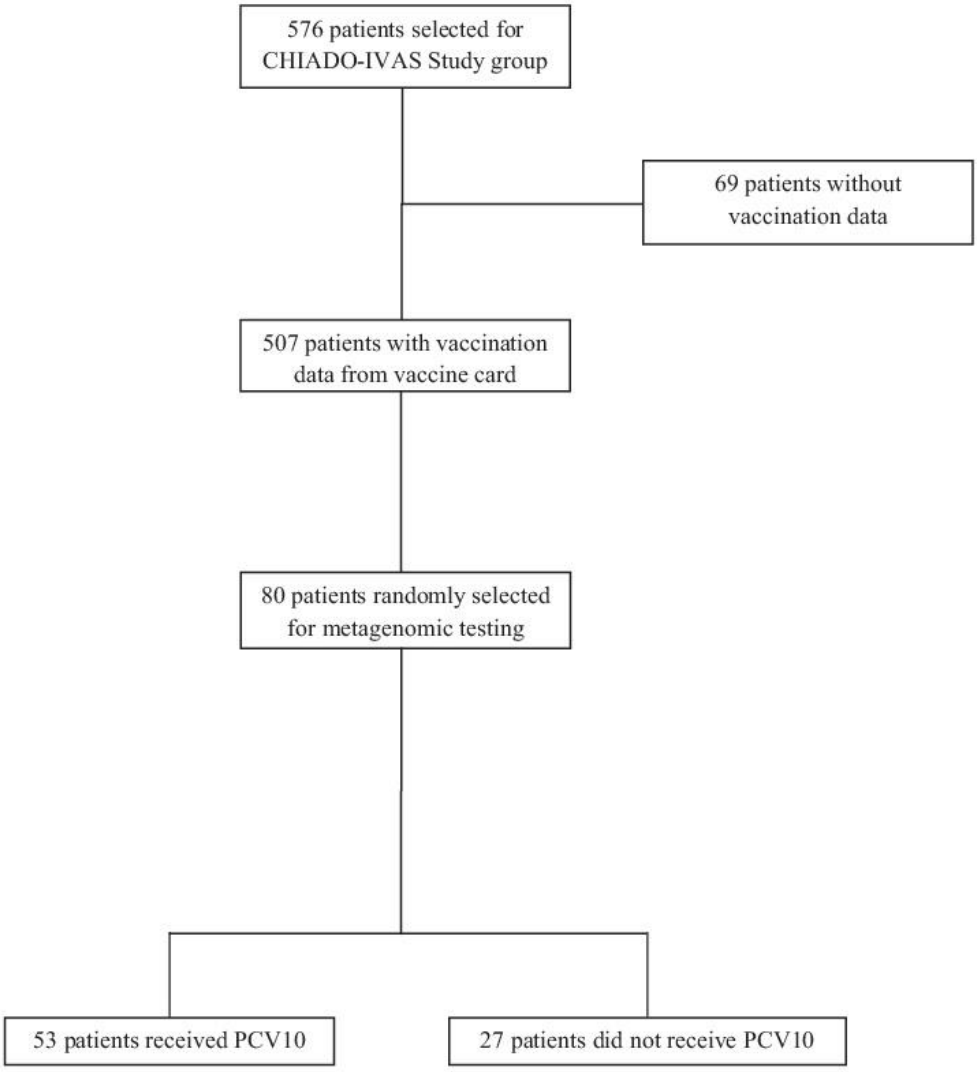

Fig. 2. Flow-chart of the included and excluded cases in this study.

Table 1
Comparison of clinical characteristics, rates of detection of RNA transcripts and 16S DNA between PCV10-vaccinated and unvaccinated patients.

	Vaccinated patients (n = 53)	Unvaccinated patients (n = 27)	P
RNA transcripts			
S. pneumoniae	15 (28.3%)	12 (44.4%)	0.149
S. aureus	53 (100%)	27 (100%)	–
H. influenzae	51 (96.2%)	26 (96.3%)	1
M. catarrhalis	47 (88.7%)	24 (88.9%)	1
16SrRNA DNA			
S. pneumoniae	25 (47.2%)	13 (48.1%)	0.934
S. aureus	41 (77.4%)	21 (77.8%)	0.966
H. influenzae	53 (100%)	27 (100%)	–
M. catarrhalis	53 (100%)	27 (100%)	–
Clinical characteristics			
Sex Male	20 (37.7%)	11 (40.7%)	0.794
Age in months, median (p25th-p75th)	9.2 (7.9–13.9)	10.8 (8.2–16.8)	0.228
History of fever[a]	41 (78.8%)	22 (81.5%)	0.782
History of running nose	47 (88.7%)	25 (92.6%)	0.710
History of hoarseness	17 (32.1%)	8 (29.6%)	0.8231
History of otalgia	3 (5.7%)	1 (3.7%)	1
History of wheezing	20 (37.7%)	13 (48.1%)	0.371
History of dyspnea	16 (30.2%)	5 (18.5%)	0.262
History of chest retraction	5 (9.4%)	0 (0%)	0.161

[a] N: 79 patients due to missing data.

Table 2
Comparison of median (interquartile interval) RNA transcripts counts and 16S rRNA DNA reads between PCV10-vaccinated and unvaccinated patients.

	Vaccinated patients (n = 53)	Unvaccinated patients (n = 27)	P
RNA transcripts			
S. pneumoniae	0 (0–1.49)	0 (0–3.43)	0.112
S. aureus	8 (6.25–9.42)	5.62 (4.58–7.08)	0.001
H. influenzae	11.47 (6.53–19.04)	13.98 (6.3–17.14)	0.695
M. catarrhalis	5.52 (4.07–6.46)	5.58 (4.26–6.26)	0.819
16S DNA			
S. pneumoniae	0 (0–0.02)	0 (0–0.03)	0.982
S. aureus	0.01 (0.01–0.05)	0.01 (0.01–0.2)	0.508
H. influenzae	0.45 (0.24–14.82)	1.24 (0.23–11.51)	0.927
M. catarrhalis	13.44 (0.82–53.24)	37.5 (11.05–54.49)	0.191

pneumococci, as suggested by previous mathematical modelling [20]. *Streptococcus pneumoniae* have sensor-kinase signal systems that detect environmental parameters that allow changes in their genetic programs in response [21]. Therefore, rather than solely affecting carriage rates, pneumococcal vaccination may promote changes in the biological behavior of colonizing pathogenic bacteria in the nasopharynx by changing environmental characteristics.

Herein, we found that carried pneumococci in PCV10-vaccinated children have a lower metabolic rate compared to those

Table 3
Comparison of the ratio between RNA transcripts counts and 16S rRNA DNA reads between PCV10-vaccinated and unvaccinated patients who had carriage of each bacterium detected by metagenomic analysis.

	Vaccinated patients	Unvaccinated patients	p
S. pneumoniae (n = 38)	0 (0–47.83)	126 (22.75–218.41)	0.004
S. aureus (n = 62)	292.87 (82.74–733.47)	253.99 (140.35–594.58)	0.982
H. influenzae (n = 80)	18.85 (0.55–47.21)	7.5 (0.66–36.83)	0.590
M. catarrhalis (n = 80)	0.21 (0.07–7.39)	0.12 (0.07–0.52)	0.359

in unvaccinated children (Table 3). This finding corroborates the lower invasive potential of colonizing pneumococci following PCV use [19]. The reduction in pneumococcal activity in vaccinated children may represent an adaptive response from this bacterium in a hostile environment created by the activated immune system. Also, unvaccinated children had a positive correlation between bacterial load (16S rRNA DNA reads) and mRNA counts, indicating that carried bacteria were in an active replicating state. Vaccinated children, however, had a poor correlation, which might represent inhibition of replication or even bactericidal activity of the immune system.

Vaccinated children also presented a poor correlation between bacterial load (16S rRNA DNA reads) and mRNA counts from *H. influenzae*. The effect of PCV10 on nasopharyngeal carriage of *H influenzae* has not been elucidated so far. Prymula et al. (2009) reported a decrease in the rates of carriage after the booster dose of a NTHi Protein D conjugated pneumococcal vaccine, whereas Vesikari et al. (2016) found no difference in the carriage rates between PCV10-vaccinated and unvaccinated children [8,22]. In Brazil, higher rates of colonization by *H influenzae* were detected in PCV10 vaccinated children [18]. In our study, the carriage rates by this bacterium were extremely high both in vaccinated and unvaccinated children, so no differences were found in carriage rates or bacterial load. However, we hypothesize that PCV10 yields an antigen-specific response that suppresses the bacterial metabolic activity or leads to bacterial killing, thereby creating the poor correlation between carriage and transcription for *H. influenzae* in vaccinated children.

We found higher mRNA counts from *S. aureus* in children who received PCV10, indicating that although the carriage rates were similar in vaccinated and unvaccinated children, colonizing strains in vaccinated children had a higher metabolic profile. As we found lower metabolic activity of *S. pneumoniae* in vaccinated children, this finding corroborates the negative association between nasopharyngeal colonization by *S. pneumoniae* and *S. aureus* [5,6]. Regarding nasopharyngeal carriage of *M. catarrhalis*, we found no difference on the carriage rates or metabolic activity of this bacterium between PCV10 vaccinated and unvaccinated children. Differences in mRNA counts among children who received different number of doses of PCV10 may have been caused by changes in nasopharyngeal bacterial interactions due to vaccination, as dynamic interactions between different colonizers have been reported [23]. Indeed, clustering was observed between mRNA counts from *S. pneumoniae* and 16S DNA reads and mRNA counts from *M. catarrhalis* (Fig. 4).

The limitations of this study should be noted. The group of vaccinated children was not homogenous, as children received different number of doses of PCV10. However, we found no effect of the number of doses of PCV10 on the rates of detection of RNA transcripts or bacterial carriage (16S DNA), as assessed by logistic regression. Similarly, there was no effect of the number of doses of PCV10 on the bacterial load or on the metabolic rate for any of the studied bacteria. Therefore, the differences in the vaccination schemes did not significantly affect the outcome variables from

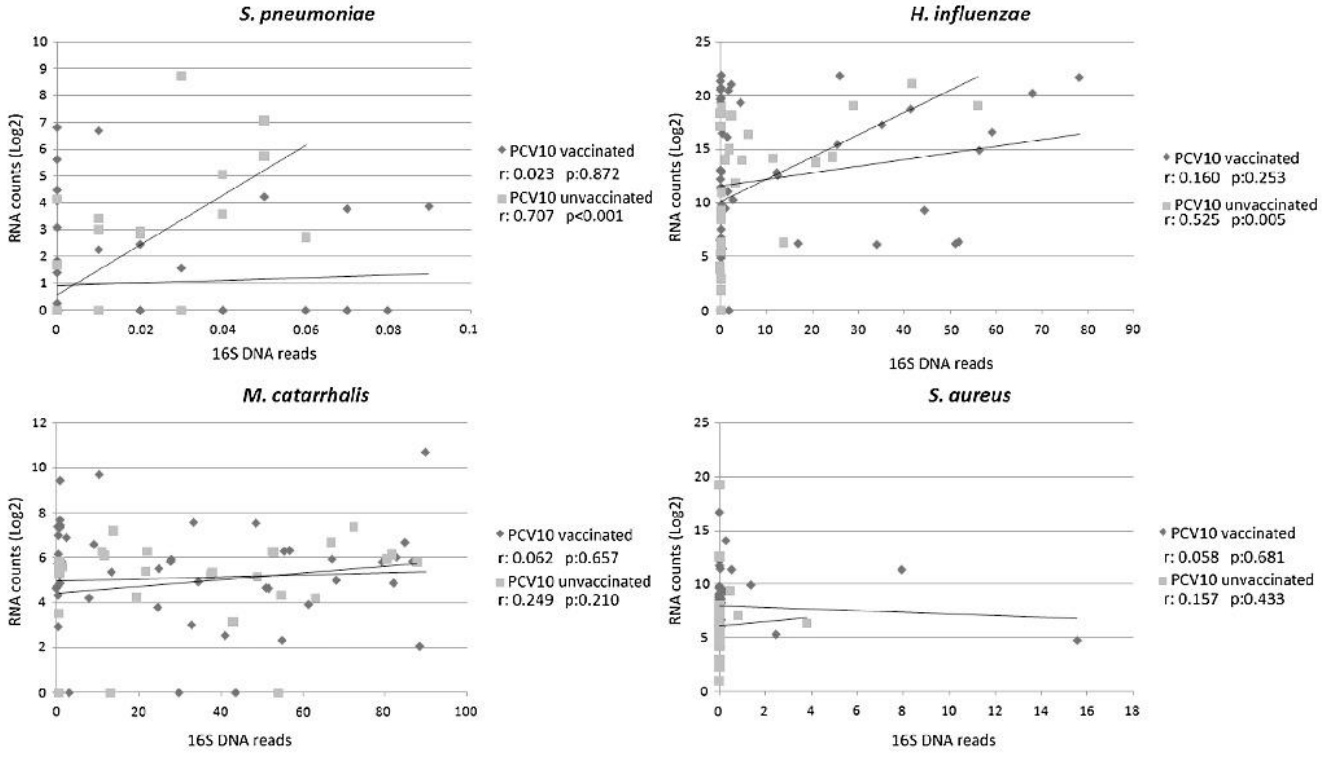

Fig. 3. Correlation between mRNA counts and 16S rRNA DNA reads from *Streptococcus pneumoniae, Haemophilus influenzae, Moraxella catarrhalis* and *Staphylococcus aureus* among PCV10 vaccinated and unvaccinated children.

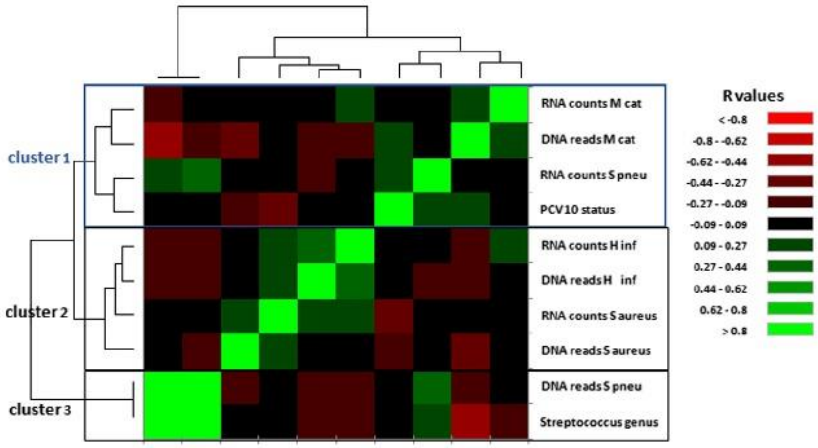

Fig. 4. Dynamic interplay between pathogenic genera and species at both RNA and DNA level following PCV10 vaccination. RNA and DNA levels of respiratory pathogens were quantified by nCounter digital transcriptomics and 16S metagenomics, respectively. Results were clustered according to Spearman's correlation and shown as heatmap (red = negative, green = positive). The blue cluster (1) indicates vaccination status is strongly correlated to *S. pneumoniae* RNA levels and *M. catarrhalis* DNA levels, whereas *H. influenzae* and *S. aureus* cluster together at both RNA and DNA level. Cluster 3 confirms *S. pneumoniae* DNA levels (carriage) are independent from its RNA levels, but are strongly correlated to the DNA frequency of the entire Streptococcus genus. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

this study. Finally, although carriage rates described herein were extremely high for some pathogenic bacteria, high carriage rates for the studied bacteria have been described elsewhere [23–25]. Furthermore, we applied a highly sensitive method (and still specific, as shown in Fig. 1), so higher carriage rates were expected when compared to previous reports.

In conclusion, vaccination with PCV10 did not affect carriage rates or the bacterial load of colonizing *S. pneumoniae, H. influenzae, M. catarrhalis*, and *S. aureus*. However, we found lower metabolic activity of *S. pneumoniae* in vaccinated children, paralleled by higher RNA counts for *S. aureus*. Furthermore, only unvaccinated children presented a positive correlation between bacterial load and mRNA transcripts for *S. pneumoniae* and *H. influenzae*, indicating an immune-mediated suppressive or bactericidal effect in vaccinated children. Altogether, our data demonstrates that pneumococcal conjugate vaccination alters pathogen RNA/DNA ratio in the nasopharyngeal microbiome, which might represent a more sensitive readout for vaccine follow-up, as compared to nasopharyngeal carriage.

Conflicts of interest

None to declare.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2017.06.048.

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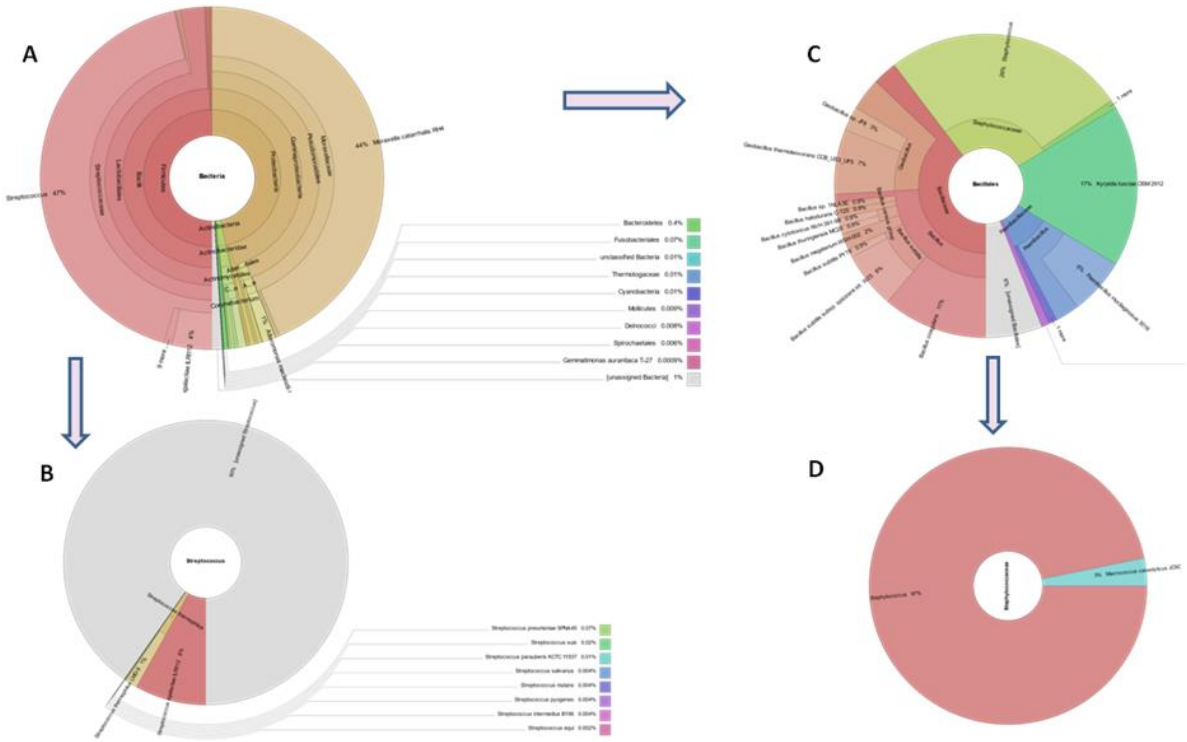

Supplementary Figure 1. Microbiome visualization of a representative sample

Following DNA extraction from nasopharyngeal aspirate, PCR amplification of V3 and V4 regions of the16S gene and next-gen sequencing (Illumina), metagenomic analysis of ARI patient sample 419 was performed using Kraken software and visualized by Krona charts (both on BaseSpace, Illumina). A) Total bacterial reads are predominated by Streptococcus genus (47%) and *Moraxella catarrhalis* species (44%). B) Focusing on the Streptococcus genus, the predominant species were *Streptococcus agalactiae*, *Streptococcus thermophilus* and *Streptococcus pneumoniae* (2%, 1% and 0.07% of total bacterial reads, respectively). C) The order Bacillales represented only 0.1% of total bacterial reads, of which the Staphylococcus genus represented a minority (26%, corresponding to 0.026% of total bacterial reads). D) Within the Staphylococcus genus, no reads for *Staphylococcus aureus* species were detected in this sample.

VIII. Artigo 5

ANTIBODY RESPONSES AGAINST STREPTOCOCCUS PNEUMONIAE, HAEMOPHILUS INFLUENZAE AND MORAXELLA CATARRHALIS IN CHILDREN WITH ACUTE RESPIRATORY INFECTION WITH OR WITHOUT NASOPHARYNGEAL BACTERIAL CARRIAGE

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ORIGINAL ARTICLE

Antibody responses against *Streptococcus pneumoniae, Haemophilus influenzae* and *Moraxella catarrhalis* in children with acute respiratory infection with or without nasopharyngeal bacterial carriage

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ABSTRACT

Background: We studied Immunoglobulin G (IgG) antibody responses against *Streptococcus pneumoniae, Haemophilus influenzae* and *Moraxella catarrhalis* in young children with acute viral type respiratory infection and analyzed the findings in a multivariate model including age, nasopharyngeal carriage of the tested bacteria and pneumococcal vaccination.

Methods: We included 227 children aged 6–23 months with acute respiratory infection. Nasopharyngeal aspirates were tested for bacterial carriage through detection of messenger RNA (mRNA) transcript with nCounter analysis. Acute and convalescent serum samples were tested for IgG antibody response against eight pneumococcal proteins, three proteins from *H. influenzae* and five proteins from *M. catarrhalis* in a fluorescent multiplex immunoassay.

Results: A two-fold or greater increase in antibodies to *S. pneumoniae, H. influenzae* and *M. catarrhalis* was detected in 27.8, 9.7 and 14.1%, respectively. Nasopharyngeal carriage of each of the studied bacteria was not associated with antibody response detection against each respective bacterium. Furthermore, neither age nor pneumococcal vaccination were independently associated to detection of antibody response against the studied bacteria. Children who carried *H. influenzae* had higher frequency of colonization by *M. catarrhalis* (175 [80.3%] vs. 2 [22.2%]; $p < .001$) than those without *H. influenzae*. Also, children with acute otitis media tended to have higher frequency of antibody response to *S. pneumoniae*.

Conclusion: Nasopharyngeal colonization by *S. pneumoniae, H. influenzae* and *M. catarrhalis* did not induce significant increases in antibody levels to these bacteria. Carriage of pathogenic bacteria in the nasopharynx is not able to elicit antibody responses to protein antigens similar to those caused by symptomatic infections.

KEYWORDS
Bacterial infection
Immune response
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Introduction

Nasopharyngeal colonization is a dynamic process in which commensal bacteria interact with the host and other carried microbiota [1]. It is a fundamental step for the development of bacterial invasive infection [1]. *Streptococcus pneumoniae, Haemophilus influenzae* and *Moraxella catarrhalis* are commonly carried bacteria in childhood [2] and have been implicated in the development of complications following acute respiratory infection (ARI), such as acute otitis media (AOM) [3,4] and community-acquired pneumonia (CAP) [5]. Furthermore, nasopharyngeal bacterial colonization has also been associated with occurrence of viral ARI [6].

Antibody responses to species-specific protein antigens have been reported following colonization, with increases detected for *S. pneumoniae* and *H. influenzae* [7–10] and decreases detected for *M. catarrhalis* [11]. In this context, the detection of increases in basal antibody levels caused by nasopharyngeal colonization during viral ARI in experimental studies may lead to a misdiagnosis of bacterial infection. Detection of antibody responses (defined as an increase $\geq$ two-fold in the antibody levels) against protein antigens from these bacteria, however, has only been associated with symptomatic respiratory infections so far [12–15].

We compared the frequency of antibody responses against *S. pneumoniae, H. influenzae* and *M. catarrhalis* in paired serum samples from children with ARI who did or did not carry the evaluated bacteria in a model controlled by 10-valent pneumococcal conjugate vaccine (PCV10) use and age. Furthermore, we evaluated whether children who developed probable bacterial complications had higher frequency of antibody response against the studied bacteria.

Materials and methods

Patients and samples

We included patients from a prospective cohort study (CHIADO-IVAS) which evaluated children with viral type ARI seen at the Pediatric Emergency Department of the Federal University of Bahia Hospital, in Salvador, Northeastern Brazil, between September 2009 and October 2013 [16]. Inclusion criteria comprised children aged from 6–23 months who presented either fever, sneeze, running nose, nasal blockage or cough for up to seven days. Children with signs and symptoms that are commonly attributed to viral infections but may also be caused by bacteria were excluded from this study (such as children with tonsillar exudates). Fever was defined as axillary temperature $>37.4\,^{\circ}C$ and tachypnoea as respiratory rate (RR) ≥ 50 breaths/min among children aged 6–11 months and RR ≥ 40 breaths/min in children from 12 months of age upwards. Nutritional status was evaluated using the software Anthro, version 3.2.2; malnutrition, overweight and obesity were defined as z-score for weight-for-height index, respectively, lower than -2, higher than 2 and higher than 3, by using the World Health Organization (WHO) standard. In Salvador, Brazil, the use of PCV10 (Synflorix, GlaxoSmithKline Biologicals, Rixensart, Belgium) was universally introduced on July 2010 for children aged <2 y. Clinical data was recorded in a standardized form and the vaccine card was checked for PCV administration with the respective number of doses, by a member of the research team. Written informed consent was obtained from legal guardians before recruitment and the study was approved by the Ethics Committee of the Federal University of Bahia.

Development of complications was assessed at the follow-up visit 2–4 weeks after study admission and included occurrence of pneumonia (defined as cough or fever along with difficulty in breathing and pulmonary infiltrates, consolidation or pleural effusion in the chest radiograph, as assessed by the pediatrician), acute otitis media (defined as earache along with tympanic alterations at otoscopy) and hospitalization.

Nasopharyngeal aspirate (NPA) samples were collected from included children upon the first evaluation (enrolment) using a standardized procedure: the distance from the entrance of the nostril to the nasopharynx was estimated by measuring the distance between the entrance of the nostril and the ear lobe; an aseptic plastic sputum catheter was inserted into the nostril until reaching the nasopharynx; negative pressure was applied and approximately 2 mL of nasal secretions were collected and placed in a sterile tube with 1 mL of NucliSens Lysis Buffer (Biomeriux, Boxtel, The Netherlands). NPAs from healthy adult volunteers were used as controls for laboratory tests. The collection was performed in a supine position when 2 mL of isotonic saline was instilled into each nostril and immediately aspirated into a specimen trap by inserting a flexible plastic suction catheter. After washing each nostril, 1 mL of saline was aspirated through the catheter and placed in a sterile tube with 1 mL of NucliSens Lysis Buffer.

Acute serum samples were collected upon recruitment and convalescent serum samples were collected on the follow-up visit. Then, they were stored at $-80\,^{\circ}C$

until laboratory testing, after the collection period was finished.

Laboratory procedures

Fluorescent multiplex immunoassay with Luminex Technology

Fluorescent multiplexed bead-based immunoassay (FMIA) was used to quantify the levels of antibodies against protein antigens from *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* using Luminex xMAP® technology (Thermo Fisher Scientific Co., Waltham, MA). This assay included eight recombinant proteins from *S. pneumoniae* (pneumolysin [Ply], choline binding protein A [CbpA], pneumococcal surface protein A families 1 and 2 [PspA 1 and 2], pneumococcal choline binding protein A [PcpA], pneumococcal histidine triad protein D [PhtD], serine/threonine protein kinase [StkP-C, SP1732-3] and protein required for cell wall separation of group B streptococcus [PcsB-N, SP2216-1]), three recombinant proteins from *H. influenzae* (NTHi Protein D, NTHi0371-1, and NTHi0830) and five recombinant proteins from *M. catarrhalis* (MC Omp CD, MC_RH4_2506, MC_RH4_1701, MC_RH4_3729-1 and MC_RH4_4730). Nine bead sets were created using the aforementioned proteins in the following combination: Ply, CbpA, PcpA, PhtD, StkP-C and PcsB-N were conjugated in one bead region each; PspA1 and A2 were conjugated in the same bead region and all *H. influenzae* and *M. catarrhalis* proteins were conjugated in one bead region per bacterial species [17].

The pneumococcal reference serum 007 was included on each plate as a standard to quantify antibodies against pneumococcal proteins. True duplicates were used throughout the procedure and their fluorescence readings were averaged. The presence of serological response was defined as a $\geq$ two fold increase in the antibody levels for Immunoglobulin G (IgG) against Ply, CbpA, PspA 1 and 2, PhtD, StkP-C or PcsB-N, an $\geq$1.5 fold increase in the antibody levels for IgG against PcpA or an increase in antibody levels $\geq$ two-fold against antigens from *H. influenzae* and *M. catarrhalis,* based on a previous validation study [14].

nCounter analysis

An amount equivalent to 10–50 ng of total RNA was extracted using RNeasy Kit (Qiagen's, Hilden, Germany) and was subsequently hybridized using probes against *S. pneumoniae, H. influenzae* and *M. catarrhalis*. Then, nasopharyngeal samples were simultaneously tested for colonizing bacteria using the NanoString nCounter gene expression system (NanoString Technologies, Seattle, WA [18]. This system allows capture and counts of individual messenger RNA (mRNA) transcripts. The detection of mRNA transcripts as a method to evaluate nasopharyngeal microbiota has been extensively tested in a previous study by Fukutani et al. [18]. In that study, a high agreement was obtained in the comparison of the results of nCounter and real time polymerase chain reaction (PCR) for detection of respiratory syncytial viruses (RSV) A and B and the test was able to detect bacterial RNA in healthy children, therefore demonstrating its ability to detect nasopharyngeal colonizing agents.

Laboratory tests were performed at the Laboratory for Clinical and Epidemiological Virology, Rega Institute for Medical Research, KU, in Leuven, Belgium. The raw data were processed using the software nSolver 2.0 (Nanostring Technologies) Nanostring Norm R package, in which three factors were sequentially corrected: background correction (negative control), technical variation between batches (positive control RNA) and RNA content by adjusting the counts geometric mean for the 15 housekeeping genes. The Nanostring counts were then normalized using logarithmic transformation (base 2).

Statistical analysis

Categorical variables were compared using chi-square or Fisher's exact test as appropriate and continuous variables were evaluated using Mann-Whitney U as they presented non-parametric distribution. Logistic regression models were performed using detection of antibody responses against *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* as dependent variables, respectively and age, PCV10 and nasopharyngeal carriage of the aforementioned bacteria as independent variables. All statistical tests were two tailed, with a significance level of .05. The software SPSS (SPSS, Chicago, IL,version 9.0) was used for the statistical analyses.

Results

We included 227 patients (Figure 1). The median age was 10.9 months (25–75th percentiles: 8.5–14.8 months) and 112 (49.3%) were males. PCV10 use was reported in 134 (59%) patients. Serologic response against *S. pneumoniae, H. influenzae* and *M. catarrhalis* was found in 63 (27.8%), 22 (9.7%) and 32 (14.1%) of the evaluated patients. Nasopharyngeal carriage of *S pneumoniae, H. influenzae* and *M. catarrhalis* was found in 63 (27.8%),

218 (96%) and 177 (78%) of the patients. Simultaneous colonization by *S pneumoniae* and *H. influenzae* or *M. catarrhalis* was found in 59 (26%) and 53 (23.3%) of the patients, respectively; simultaneous colonization by *H. influenzae* and *M. catarrhalis* was found in 175 (77.1%) of the evaluated patients. The baseline characteristics from the children included in this study are shown in Table 1.

Interaction between commensal bacteria in the nasopharynx

Children who carried *H. influenzae* had higher frequency of carriage of *M. catarrhalis* than children who were not colonized by that bacterium (175/218[80.3%] vs. 2/9 [22.2%]; $p < .001$). Similarly, children who carried *M. catarrhalis* had higher frequency of colonization by *H. influenzae* than children who did not carry *M. catarrhalis* (175/177 [98.9%] vs. 43/50 [86%] , $p < .001$). There was no difference in the rates of colonization by either *H. influenzae* (59/63[93.7%] vs. 159/164 [97%], $p = .267$) and *M. catarrhalis* (53/63 [84.1%] vs. 124/164 [75.6], $p = .166$) between children with or without pneumococcal carriage.

Effect of bacterial carriage on basal antibody levels

There was no difference in the basal antibody levels against protein antigens from either *S. pneumoniae, H. influenzae* and *M. catarrhalis* between children who did or did not present pneumococcal carriage, as shown in Table 2. Children who carried *H. influenzae* had higher levels of basal antibodies against *M. catarrhalis* (median [25–75th percentiles]: 183 [91.25–356.5] vs. 63 [33–150]; $p = .004$) compared to those without *H. influenzae*. There was no difference in the basal levels of antibodies against the studied bacteria between children who did or did not carry *M. catarrhalis* (data not shown). Lower concentrations of IgG against protein antigens from *S. pneumoniae* were found in children who received PCV10, as shown in Table 3.

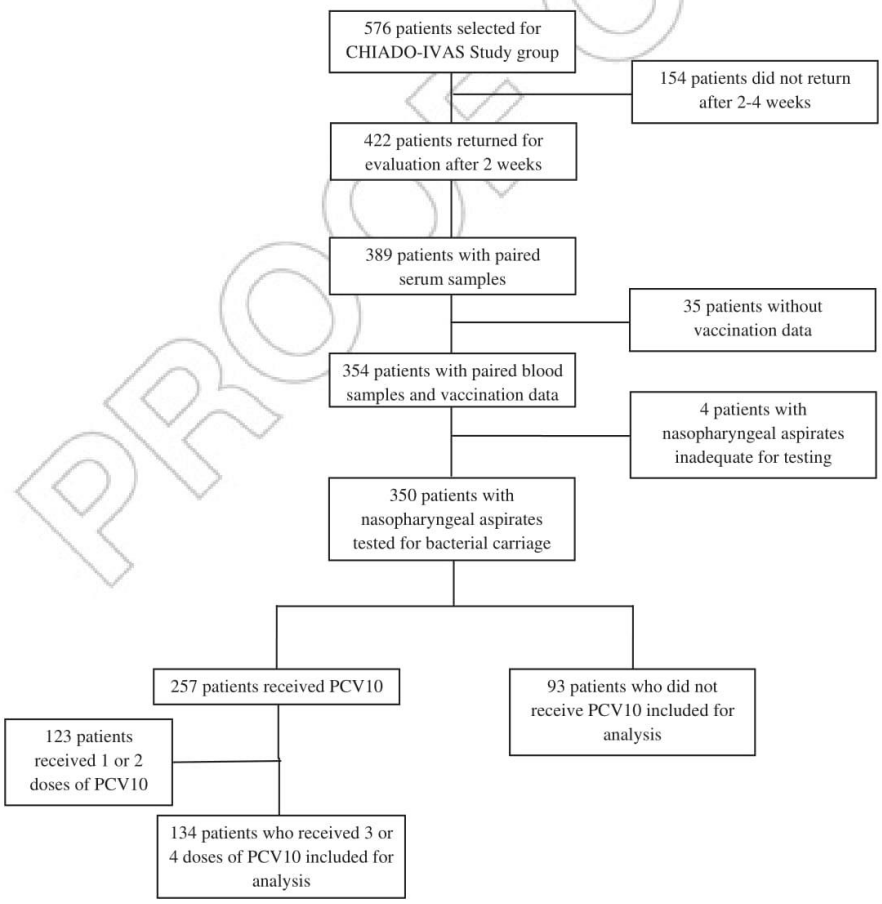

Figure 1. Flow-chart of the included and excluded cases in this study.

Effect of bacterial carriage on the frequency of antibody responses

Pneumococcal carriage had no effect in the rates of antibody response against protein antigens from *S. pneumoniae, H. influenzae* and *M. catarrhalis* (Table 4). Furthermore, there was no difference on the rates of antibody response against *H. influenzae* and *M. catarrhalis* between children who did or did not carry these bacteria (22/218 [10.1%] vs. 0/9 [0%]; $p = .605$ and 21/177 [11.9%] vs. 11/50 [22%]; $p = .069$), respectively. There was no difference in the rates of antibody response against protein antigens from *S. pneumoniae* between children who did or did not carry either *H. influenzae* or *M. catarrhalis* (data not shown)

Table 1. Clinical features from the 227 children with acute respiratory infection included in this study.[a]

Clinical characteristics	Frequency	Duration (days)
Vaccination		
Pneumococcal (PCV10)	134 (59%)	
H. influenzae type b	225 (99.1%)	
History		
Fever[b]	187 (82.4%)	3 (1–4)
Cough	200 (88.1%)	3 (2–5)
Sneeze	187 (82.4%)	3 (2–4)
Running nose	199 (87.7%)	3 (2–5)
Wheeze	89 (39.2%)	2 (1–3)
Hoarseness	72 (31.7%)	2 (1–3)
Dyspnoea	52 (22.9%)	2 (1–4)
Earache[b]	13 (5.8%)	3 (2–4.5)
Thoracic retraction	18 (7.9%)	2 (1–3.25)
Physical examination		
Malnutrition	3 (1.3%)	
Overweight and obesity	26 (11.5%)	
Tachypnoea[b]	53 (23.6%)	
Fever[b]	93 (41.9%)	
Ronchi	80 (35.2%)	
Wheezing	25 (11%)	
Crackles	21 (9.3%)	
Complications[c]		
Overall	50 (22.8%)	
Acute otitis media	12 (5.5%)	
Pneumonia	25 (11.4%)	
Hospitalization	21 (9.6%)	

[a]Data presented as median (25–75th percentiles).
[b]The denominator was not 227 because there was some information missing.
[c]The denominator was 219 patients who returned for complication assessment.

We performed a multivariate analysis to evaluate factors associated with antibody responses against *S. pneumoniae, H. influenzae* and *M. catarrhalis* among our patients (Table 5). Neither age, nor nasopharyngeal carriage nor PCV10 vaccination was independently associated with detection of antibody responses against the evaluated bacteria.

Effect of detection of antibody responses and basal antibody levels on frequency of complications

Children who developed acute otitis media during the follow-up had a higher frequency of antibody response against *S. pneumoniae,* as shown in Table 6, though statistical significance was not reached. Community-acquired pneumonia did not induce increase in antibody responses against the studied bacteria. There was no difference between the development of complications and use of PCV10 or nasopharyngeal colonization by either *S. pneumoniae, H. influenzae* or *M. catarrhalis* (data not shown).

Discussion

Our study demonstrates that nasopharyngeal colonization by either *S. pneumoniae, H. influenzae* or *M. catarrhalis* is not associated with antibody responses against the aforementioned bacteria in a model controlled by age and use of PCV10. Children who developed AOM had a higher frequency of antibody response against *S. pneumoniae,* although there was no difference in the rates of colonization by this bacterium.

Increases in the antibody levels against pneumococcal proteins following colonization by *S. pneumoniae* have been previously identified in prospective cohort studies [8–10,19]. This increase in anti-protein IgG induced by pneumococcal colonization, however, was not significant enough to allow the detection of an antibody response in a cohort of 36 children followed for the first two years

Table 2. Comparison of antibody levels in the first serum sample between children with acute respiratory infection who did or did not carry *S. pneumoniae*.

	Pneumococcal carriage ($n = 63$)	No pneumococcal carriage ($n = 164$)	p value
Ply[a]	78 (23–254)	82 (20–270)	.910
CbpA[a]	99 (6–1009)	58 (7–656.25)	.678
PspA 1 and 2[a]	31 (6–260)	21.5 (6–129.25)	.217
PcpA[a]	642 (111–1381)	397.5 (107.5–1037.25)	.287
PhtD[a]	25 (8–333)	27.5 (8.25–230)	.908
StkP-C[a]	26 (10–143)	26.5 (10–91)	.578
PcsB-N[a]	79 (11–1317)	149.5 (14.25–880.25)	.973
Haemophilus influenzae[b]	227 (110–417)	236.5 (129–447)	.531
Moraxella catarrhalis[b]	187 (89–379)	172.5 (83.5–336.25)	.632

[a]Levels of IgG expressed as a concentration in relation to the standard serum 007.
[b]Levels of IgG expressed as median fluorescence index (MFI).

of life [15]. Herein, we demonstrated the detection of antibody response against pneumococcal antigens in almost 30% of children with ARI, which was not associated with current colonization by this bacterium. On the other hand, detection of antibody responses against protein antigens from *S. pneumoniae* have been described in the context of recognized bacterial respiratory infections, such as AOM [12] and CAP [14]. Importantly, the occurrence of increases higher than two-fold in the anti-protein antibody levels has good sensitivity and specificity (> 90%) for pneumococcal pneumonia [14]. Altogether, these data suggest that the immune response elicited by symptomatic bacterial infections is different from the immune response that occurs during bacterial colonization, in which case the increase in IgG is brief and of lower magnitude.

We found that children with AOM had higher frequency of antibody response against *S. pneumoniae* than children who did not develop AOM. Importantly, Oliveira et al. [16], in a study including 422 patients from the CHIADO-IVAS cohort, reported that the use of PCV10 was inversely associated with development of AOM [16]. As the use of PCV reduces the incidence of AOM caused by the pneumococcus [20], it is probable that the excess cases of AOM found in that study were caused by *S. pneumoniae*. Herein, however, the evaluated study group was smaller due to the availability of samples for serologic analysis. Consequently, no association was found between use of PCV10 and development of complications. Indeed, the sample size required for the detection of a statistically significant difference of the same magnitude as the one reported herein (calculated as 304 patients, considering that the power of the study was 80%, the alfa error 5% and that the two tested proportions were inferior to 25% and higher than 40%, respectively) is higher than the one available for this study [21]. Therefore, we suppose that children with AOM had higher rate of infection by *S. pneumoniae*, which did not reach statistical significance due to our sample size. We also found low rates of antibody response against *S. pneumoniae* and *H. influenzae* among children who

Table 3. Comparison of antibody levels in the first serum sample between PCV10-vaccinated and unvaccinated patients.

	Vaccinated ($n = 134$)	Unvaccinated ($n = 93$)	p value
Ply[a]	64 (17–259)	114 (25.5–267.5)	.102
CbpA[a]	27.5 (5–509.25)	281 (15–1124)	.001
PspA 1 and 2[a]	21 (5–105)	35 (9.5–225.5)	.004
PcpA[a]	362 (96–905.5)	588 (166–1481)	.022
PhtD[a]	20.5 (7–233.75)	40 (11–283)	.077
StkP-C[a]	21 (8.75–73.5)	40 (13–117)	.031
PcsB-N[a]	62.5 (11–844)	232 (15.5–1167.5)	.053
Haemophilus influenzae[b]	325.5 (176–555)	157 (70.5–250)	<.001
Moraxella catarrhalis[b]	172.5 (91.25–346.5)	188 (77–336.5)	.890

[a]Levels of IgG expressed as a concentration in relation to the standard serum 007.
[b]Levels of IgG expressed as median fluorescence index (MFI).

Table 4. Comparison of the frequency of antibody responses against protein antigens from *S. pneumoniae, H. influenzae* and *M. catarrhalis* between children who were or were not colonized by *Streptococcus pneumoniae*.

	Pneumococcal carriage ($n = 63$)	No pneumococcal carriage ($n = 164$)	p value
Ply	5 (7.9%)	17 (10.4%)	.580
CbpA	1 (1.6%)	14 (8.5%)	.074
PspA 1 and 2	3 (4.8%)	15 (9.1%)	.411
PcpA	10 (15.9%)	27 (16.5%)	.914
PhtD	7 (11.1%)	16 (9.8%)	.762
StkP-C	6 (9.5%)	14 (8.5%)	.814
PcsB-N	5 (7.9%)	19 (11.6%)	.423
Streptococcus pneumoniae	15 (23.8%)	48 (29.3%)	.411
Haemophilus influenzae	5 (7.9%)	17 (10.4%)	.580
Moraxella catarrhalis	10 (15.9%)	22 (13.4%)	.634

Table 5. Multivariate analysis of factors associated with detection of antibody responses against *Streptococcus pneumoniae, Haemophilus influenzae* and *Moraxella catarrhalis*in children with acute respiratory infection.

	Antibody response against *S. pneumoniae*		Antibody response against *H. influenzae*		Antibody response against *M. catarrhalis*	
	OR (95%CI)	p value	OR (95%CI)	p value	OR (95%CI)	p value
Age	1 (0.998–1.002)	.939	1.001 (0.997–1.005)	.591	1 (0.997–1.003)	.894
Carriage of *S. pneumoniae*	0.767 (0.385–1.525)	.449	0.710 (0.243–2.073)	.531	1.265 (0.541–2.954)	.588
Carriage of *H. influenzae*	0.827 (0.186–3.685)	.803	64237981.67 (<0.001–>1000,000,000)	.999	0.464 (0.101–2.142)	.325
Carriage of *M. catarrhalis*	0.823 (0.398–1.699)	.598	6.153 (0.791–47.884)	.083	0.534 (0.223–1.282)	.160
Pneumococcal vaccination[a]	1.409 (0.768–2.587)	.268	2.214 (0.820–5.980)	.117	1.582 (0.703–3.558)	.268

[a]Pneumococcal vaccination: PCV10 3–4 doses.

Table 6. Comparison of the frequency of antibody response against *Streptococcus pneumoniae, Haemophilus influenzae* or *Moraxella catarrhalis* between children who did or did not develop complications.

	Overall complications			Otitis			Pneumonia			Hospitalization		
	Yes ($n = 50$)	No ($n = 169$)	p value	Yes ($n = 12$)	No ($n = 207$)	p value	Yes ($n = 25$)	No ($n = 194$)	p value	Yes ($n = 21$)	No ($n = 198$)	p value
Antibody response against												
S. pneumoniae	14 (28%)	46 (27.2%)	.913	5 (41.7%)	55 (26.6%)	.317	4 (16%)	56 (28.9%)	.175	6 (28.6%)	54 (27.3%)	.899
H. influenzae	4 (8%)	15 (8.9%)	1	1 (8.3%)	18 (8.7%)	1	1 (4%)	18 (9.3%)	.704	4 (19%)	15 (7.6%)	.093
M. catarrhalis	9 (18%)	22 (13%)	.375	1 (8.3%)	30 (14.5%)	1	6 (24%)	25 (12.9%)	.136	5 (23.8%)	26 (13.1%)	.190

developed CAP during follow-up. It is possible that disease in these children was caused by viruses, which are currently considered the major cause of pediatric CAP [22].

No differences were found in the levels of IgG against pneumococcal proteins in the first serum sample between children who were or were not colonized by *S. pneumoniae.* However, it is important to note that this study only evaluated one episode of nasopharyngeal colonization and the basal levels of antigen-specific antibodies reflects the occurrence of cumulative previous encounters with this bacterium. For instance, lower levels of IgG against pneumococcal proteins among children who received PCV10 reflect the lower number of episodes of colonization [23] and disease [24] caused by vaccination and has been previously reported in a cohort of children with CAP [13]. Of note, higher levels of IgG against protein antigens from *H. influenzae* might have been caused by increase in colonization by this bacterium over time, which has been reported following pneumococcal vaccination [25,26].

We found a positive interaction between colonization by *H. influenzae* and *M. catarrhalis,* with higher frequency of carriage of *M. catarrhalis*in children already colonized by *H influenzae* and vice-versa. Furthermore, children who carried *H. influenzae* had higher levels of antibodies against *M. catarrhalis* in the first serum sample, which suggests a long-standing synergistic effect for carriage of these bacteria. Co-colonization by *H. influenzae* and *M. catarrhalis* is a common finding and has been reported to occur more frequently than colonization by either bacterium alone [27]. Although bacterial competition commonly occur in the nasopharynx [28,29], it has been reported that strains of *M. catarrhalis* aid *H. influenzae* to evade complement-mediated killing by secretion of outer membrane vesicles (OMVs) carrying ubiquitous surface protein (Usp) A1 and UspA2 which interact with the third component of the complement system (C3) [30]. Therefore, our data reinforce the persistent interaction between *H. influenzae* and *M. catarrhalis* in the nasopharynx.

The limitations of this study should be noted. First, the assessment of nasopharyngeal colonization was performed at only one time point, so no information of colonization on a time series was available. However, important information was drawn from the antibody levels against the studied bacteria on the first serum sample, which reflected previous episodes of colonization or infection. Second, due to limitations in resources, our sample size was inferior to that needed to assess the differences in rates of antibody responses between patients who did or did not present complications. Nevertheless, we still could observe higher rate of antibody responses against *S. pneumoniae* between children who developed AOM, which is in accordance with previously published data. Third, the diagnosis of viral-type ARI was made clinically. However, children with signs and symptoms that could be indicative of a bacterial infection were excluded from this study. Furthermore, the patients included in this study presented a high frequency of symptoms such as cough (88.1%), sneezing (82.4%) and running nose (87.7%), which associated to the age between 6–23 months is highly suggestive of viral infection. Finally, we did not evaluate the development of mucosal immune responses, which may occur in the setting of nasopharyngeal colonization. Nevertheless, current evidence suggests that the development of serologic response is not limited by mucosal immunity [8,31–34]. Therefore, this should not limit the interpretation of our results.

In conclusion, nasopharyngeal colonization activates the immune system but is not able to elicit antibody responses similar to those caused by symptomatic bacterial infections. Children with ARI who develop AOM have higher rate of antibody responses against *S. pneumoniae.*

Disclosure statement

No potential conflict of interest was reported by the authors.

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IX. Artigo 6

EFFECT OF PNEUMOCOCCAL CONJUGATE VACCINE ON THE NATURAL ANTIBODIES AND ANTIBODY RESPONSES AGAINST PROTEIN ANTIGENS FROM *STREPTOCOCCUS PNEUMONIAE, HAEMOPHILUS INFLUENZAE* AND *MORAXELLA CATARRHALIS* IN CHILDREN WITH COMMUNITY-ACQUIRED PNEUMONIA.

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Effect of Pneumococcal Conjugate Vaccine on the Natural Antibodies and Antibody Responses Against Protein Antigens From *Streptococcus pneumoniae, Haemophilus influenzae* and *Moraxella catarrhalis* in Children With Community-acquired Pneumonia

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Background: *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis* are common causative agents of respiratory infections. Pneumococcal conjugate vaccines have been introduced recently, but their effect on the natural immunity against protein antigens from these pathogens has not been elucidated.

Methods: This was an age-matched observational controlled study that evaluated the influence of 10-valent pneumococcal conjugate vaccines on the levels of antibodies and frequencies of antibody responses against proteins from *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* in serum samples of children with community-acquired pneumonia. Eight pneumococcal proteins (pneumolysin, choline-binding protein A, pneumococcal surface protein A families 1 and 2, pneumococcal choline-binding protein A, pneumococcal histidine triad protein D, serine/threonine protein kinase, protein required for cell wall separation of group B streptococcus), 3 proteins from *H. influenzae* (including protein D) and 5 *M. catarrhalis* proteins were investigated.

Results: The study group comprised 38 vaccinated children and 114 age-matched controls (median age: 14.5 vs. 14.6 months, respectively; $P = 0.997$), all with community-acquired pneumonia. There was no difference on clinical baseline characteristics between vaccinated and unvaccinated children. Vaccinated children had significantly lower levels of antibodies against 4 of the studied pneumococcal antigens ($P = 0.048$ for Ply, $P = 0.018$ for pneumococcal surface protein A, $P = 0.001$ for StkP and $P = 0.028$ for PcsB) and higher levels of antibodies against *M. catarrhalis* ($P = 0.015$). Nevertheless, the vaccination status did not significantly affect the rates of antibody responses against *S. pneumoniae*, *H. influenzae* and *M. catarrhalis*.

Conclusions: In spite of the differences that have been found on the level of natural antibodies, no effect from pneumococcal vaccination was observed on the rate of immune responses associated with community-acquired pneumonia against protein antigens from *S. pneumoniae*, *H. influenzae* and *M. catarrhalis*.

Key Words: humoral antibody response, immune factors, lower respiratory tract infection, polysaccharides, serological tests

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Streptococcus pneumoniae, Haemophilus influenzae and *Moraxella catarrhalis* are common bacteria associated with acute respiratory infections in childhood, such as community-acquired pneumonia (CAP)[1] and acute otitis media.[2] Furthermore, these bacteria also frequently colonize the nasopharyx,[3–5] which might be considered the initial step of their pathogenesis.[6] As a preventive strategy to control the burden of disease by these agents, polysaccharide-based conjugate vaccines have been introduced against *S. pneumoniae*[7] and *H. influenzae* type b.

Pneumococcal conjugate vaccines (PCV) are being increasingly implemented worldwide. As a consequence, the incidence of disease and carriage of vaccine-covered serotypes has decreased.[2,5,8] Nevertheless, an increase in colonization by nonvaccine serotypes has been identified,[2] which may represent a limitation for the use of polysaccharide-based vaccines. As a result, new protein antigens have been reported as putative vaccine candidates due to their specificity and high degree of conservation among the strains of each bacterium.[3–5]

Few studies have addressed the development of natural antibodies and the frequency of antibody responses against protein antigens from *S. pneumoniae, H. influenzae* and *M. catarrhalis*.[9–13] In addition, most of these studies were conducted before the introduction of the current polysaccharide-based vaccines (PCV and *H. influenzae* type b vaccine), which does not represent the general pediatric population anymore. We aimed to evaluate differences in the levels of natural antibodies and in the frequencies of antibody responses associated with CAP against protein antigens from *S. pneumoniae, H. influenzae* and *M. catarrhalis* in children who had been vaccinated or left unvaccinated with 10-valent PCV (PCV10).

MATERIALS AND METHODS

Study Design and Participants

This was an age-matched observational controlled study exploring the effects of vaccination with PCV10 on the levels of natural antibodies and frequency of antibody responses against protein antigens from *S. pneumoniae, H. influenzae* and *M. catarrhalis*. This study was part of the PNEUMOPAC-Efficacy AQ3

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trial (ClinicalTrials.gov NCT01200706)[14] in which 820 children aged 2–59 months old with nonsevere CAP were recruited in the Emergency Department of the Federal University of Bahia hospital in Salvador, Northeast Brazil, from November 2006 to May 2011. Inclusion criteria were report of respiratory complaints, detection of lower respiratory findings on physical examination and the presence of pulmonary infiltrate/consolidation on the chest radiograph taken on admission and read by the attending pediatrician. Exclusion criteria from this trial comprised the presence of chest indrawing or danger signs and underlying chronic diseases or immunodeficiency. Legal guardians from the included patients provided a written informed consent upon enrolment. Blood samples were collected at admission and 2–4 weeks later. After sample collection, they were immediately processed and stored at −20°C until the moment of analysis. Sample collection was performed regardless of the completion of the vaccination schedule.

The use of PCV10 (Synflorix, GlaxoSmithKline Biologicals, Rixensart, Belgium) was universally introduced in Salvador, Brazil, on July 2010 for children aged <2 years.[15] The scheduled vaccination regimen on the first year of implementation of this immunization program varied according to the age of the child. Children aged between 2 and 6 months received 3 doses of vaccine on the first year of life with a 2-month interval, with one booster dose at 12 months of age. Children aged 7–11 months who had not been previously vaccinated received 2 doses of PCV10, with a 2-month interval, along with a booster dose between 12 and 15 months of age. Finally, children aged 12–24 months only received one dose of PCV10, with no booster dose of the vaccine.[15] Every child included in the PNEUMOPAC-efficacy trial who could have received PCV10 was identified based on the age and date of enrollment, comprising a group of 104 children. Each of these children had the vaccine card checked personally by one of the researchers (I.C.B.) after the trial was completed. Any dose of this vaccine given to those children was recorded along with the date of administration in a logbook. From these data, the number of doses of PCV10 and time interval between the first and last doses of PCV10 and the collection of the first serum sample were calculated.

In this observational controlled study, the studied cases were represented by vaccinated children from the PNEUMOPAC-Efficacy trial, and the controls were age-matched unvaccinated children from the same trial. Age matching was performed as a method to control for the potential effect of age on the levels of antibodies against the studied antigens. The primary criterium for the selection of controls was the age of unvaccinated children, which should be the closest ones to the age of the vaccinated cases. The case:control relation was 1:3. Only if more than 3 suitable controls had the same age difference to the vaccinated case, the entry order on the PNEUMOPAC-Efficacy trial was used to select the children who were enrolled first as controls.

Patients with severe malnutrition, defined as Z score for weight-for-age under −3.00,[16] were excluded from this study. Nutritional evaluation was performed using "Anthro" software.

The PNEUMOPAC-Efficacy trial was approved by the Ethics Committee of the Federal University of Bahia and was conducted in accordance with the principles of the Declaration of Helsinki.

Laboratory Procedures

The concentration of antibodies against protein antigens from *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* was determined with a multiplexed bead-based serological assay with Luminex xMAP technology.[17] The test included 8 recombinant proteins from *S. pneumoniae*: pneumolysin, choline-binding protein A, pneumococcal surface protein A families 1 and 2 (PspA 1 and 2), pneumococcal choline-binding protein A, pneumococcal histidine triad protein D, serine/threonine protein kinase (StkP-C, SP1732-3, a C-terminal fragment of StkP) and protein required for cell wall separation of group B streptococcus (PcsB-N, SP2216-1, a N-terminal fragment of PcsB), 3 recombinant proteins from *H. influenzae* (NTHi Protein D—the carrier protein in PCV10, heme/hemopexin utilization protein A [NTHi0371-1], and outer membrane antigenic lipoprotein B [NTHi0830]) and 5 recombinant proteins from *M. catarrhalis* (OMP CD, Msp22 [MC_RH4_2506], extracellular solute-binding protein [MC_RH4_1701], probable membrane-bound lytic murein transglycosylase D [MC_RH4_3729-1] and Methionine-R-sulfoxide reductase [MC_RH4_4730]). Using these antigens, 9 bead sets were produced using the following combination: pneumolysin, choline-binding protein A, pneumococcal choline-binding protein A, pneumococcal histidine triad protein D, StkP-C and PcsB-N were conjugated in one bead region each; PspA 1 and PspA 2 were conjugated in the same bead region; and all *H. influenzae* and all *M. catarrhalis* proteins were conjugated in one bead region per bacterium.

For each antigen, the mean fluorescence intensity (MFI) values were obtained as an indirect measure of the immunoglobulin G concentration. Samples were assayed in duplicate and the results averaged. Acute and convalescent samples were always tested on the same plate. Furthermore, positive and negative controls were analyzed on each plate to ensure good batch-to-batch consistency.[17] All samples were analyzed at 1:400 and 1:1600 dilutions, and when necessary, further dilutions were performed.

The levels of natural antibodies were defined as the MFI readings for each studied antigen measured on the first serum sample. The presence of an antibody response was defined as an increase on the antibody levels ≥2-fold between the first and second serum samples.[18] All the samples were tested from October 2012 to January 2013.

Statistical Analysis

Categorical variables were compared using χ^2 or Fisher exact test as appropriate and continuous variables were evaluated using Mann-Whitney *U* test as they presented a nonparametric distribution. Correlation between 2 continuous variables was evaluated with Spearman correlation. Kruskal-Wallis test was used when AQ4
the association of a categorical variable with more than 2 levels and a continuous variable was evaluated. Logistic regression was performed to evaluate the effect of continuous or categorical variables on a dichotomized variable, and linear regression was used to evaluate the effect of continuous or categorical variables on a continuous variable. The statistical tests were 2-tailed, with a significance level of 0.05. The software SPSS (version 9.0) was used for the analyses.

RESULTS

Study Group

Out of the 820 patients from PNEUMOPAC-Efficacy study, this study included 38 vaccinated patients and 114 age-matched controls. Pairing between vaccinated and unvaccinated children according to age at admission was successful, and the maximum age difference between a case and its respective control was 10 days. The included unvaccinated children had similar demographic and serological characteristics when compared with the PNEUMOPAC cohort, whose data have been previously published.[19] The comparison of the baseline clinical features for vaccinated and unvaccinated children demonstrated no significant difference between the study subgroups, as shown in Table 1. T1

TABLE 1. Comparison of Baseline Characteristics for Vaccinated (Cases) and Unvaccinated (Controls) Children

	PCV10 Vaccination		
	Vaccinated (n=38)	Unvaccinated (n=114)	*P*
Sex (male)	24 (63.2%)	65 (57%)	0.506
Age (months)	14.5 (9.8–18.8)	14.6 (10–19)	0.997
Length of disease (days)*	5 (4–7.25)	6 (3–10)	0.272
Interval between samples (days)*	17.5 (16–20.25)	19 (16–21)	0.590
Fever	33 (86.8%)	103 (90.4%)	0.549
Duration of fever (days)*	3 (2–5)	4 (2–5)	0.324
Cough	35 (92.1%)	111 (98.2%)	0.102
Duration of cough (days)*	5 (3–8)	6 (4–10)	0.069
Difficulty breathing	25 (65.8%)	83 (73.5%)	0.365
Duration of difficulty breathing (days)*	3 (1.5–5)	3 (1–5)	0.739
Wheezing	12 (31.6%)	34 (29.8%)	0.838
Duration of wheezing (days)*	3 (2–4)	3 (1–5)	0.849
Vomit	16 (42.1%)	54 (47.4%)	0.573
Duration of vomiting (days)*	1 (1–3)	2 (1–3)	0.259

*Continuous variables presented as median (25th–75th percentile).

On the date of the first serum sample collection, most vaccinated children (cases) had completed the appropriate vaccination schedule. Figure 1 shows the flowchart of the included and excluded cases of this study, along with the vaccination status and the number of doses of PCV administered to each vaccinated child included herein.

Differences in the Levels of Natural Antibodies in Vaccinated and Unvaccinated Children

The comparison of the MFI values, expressing the levels of natural antibodies against protein antigens from *S. pneumoniae, H. influenzae* and *M. catarrhalis,* between age-matched vaccinated and unvaccinated children with CAP using the 1:1600 dilution factor is shown in Table 2. Overall, vaccinated children presented lower levels of antibodies against most of the tested pneumococcal antigens and higher levels of antibodies against *M. catarrhalis.* No significant difference was seen for *H. influenzae* proteins. Similar results were found when data from the 1:400 dilution was used (data not shown).

Antibody Responses Associated With CAP Against Protein Antigens From *S. pneumoniae, H. influenzae* and *M. catarrhalis* in Vaccinated and Unvaccinated Children

The comparison of the frequency of responders for each studied antigen in age-matched vaccinated and unvaccinated children is shown in Table 3. No significant differences were found in the frequency of antibody responses between vaccinated and unvaccinated children. Overall, 7 patients presented decreases ≤0.5 in the levels of antibodies against any protein from *S. pneumoniae* (vaccinated vs. unvaccinated: 2 [5.3%] vs. 5 [4.4%]; $P = 1$). None of the patients presented decreases in the antibody levels against *H. influenzae* and *M. catarrhalis.*

Effect of the Number of Doses of PCV10 and the Time Interval From the First/Last Dose of Vaccine on the Levels of Natural Antibodies and Frequency of Antibody Responses in Vaccinated Children

There was no effect of number of administered doses of PCV10 on the levels of natural antibodies against protein antigens at either 1:400 or 1:1600 dilution using Kruskal-Wallis test (data not shown). No effect of time interval between the first or last dose of PCV10 and the collection of the first serum sample on the levels of natural antibodies against protein antigens was found for *S. pneumoniae* and *M. catarrhalis*, as shown in Table 4. There was a positive association between the time interval from the first dose of PCV10 to the collection of the first serum sample and the level of natural antibodies against *H. influenzae.* However, when a linear regression was performed adjusting the aforementioned association by the age of the child upon enrollment, no effect of the time interval between the first dose of PCV10 and the collection of the first serum sample on the levels of antibodies against *H. influenzae* was detected ($P = 0.697$).

Neither the number of doses of PCV10 nor the time interval between the administration of the first/last dose of PCV10 and the collection of the first serum sample influenced the frequency of antibody responses against the studied protein antigens when evaluated using logistic regression (data not shown).

DISCUSSION

Our case-control study demonstrated that there is a significant difference in the levels of natural antibodies against protein antigens from *S. pneumoniae* and *M. catarrhalis* between age-matched children who were vaccinated with PCV10 or did not receive this vaccine. Nevertheless, there was no difference between vaccinated and unvaccinated in the frequency of antibody responders against the studied antigens upon contraction of CAP.

Of note, vaccinated children had significantly lower MFI values to 4 of the evaluated pneumococcal antigens (pneumolysin, PspA, StkP and PcsB). Also, the antibody levels against choline-binding protein A, pneumococcal choline-binding protein A and pneumococcal histidine triad protein D were lower in vaccinated children compared with unvaccinated controls, but this did not reach statistical significance. Similarly, in the study by Prevaes et al,[9] there was also a trend toward lower MFI readings in vaccinated children for the antigens Nan, Pilus A, PspA and PsaA in 24-month-old children. Conversely, Ditse et al[13] found no difference on the levels of antibodies against protein antigens between children vaccinated with PCV7 or unvaccinated. Nevertheless, the study population from Ditse et al[13] was significantly older than the one herein, which might compromise the comparison of the effect of pneumococcal vaccination due to the long-term decrease in effectiveness of PCVs after primary vaccination.[20] Accordingly, no effect of vaccination on the prevalence of overall or vaccine-serotype colonization by *S. pneumoniae* was found in the aforementioned study.[13,20] In addition, this study is the first evaluating the effect of PCV10 on the level of natural anti-protein antibodies in vaccinated and unvaccinated children. Consequently, it is possible that the greater coverage provided by PCV10 when compared with the PCVs used in previous studies might have influenced the difference in the natural levels of antibodies between vaccinated and unvaccinated children.

The lower MFI values found for vaccinated children might have been caused by the effects of PCV10 on the rate of nasopharyngeal colonization. For instance, the use of PCV10 may have reduced the overall rate of pneumococcal carriage for the studied children as it has already been reported in previous vaccine trials with other types of PCV.[21,22] In this setting, by decreasing the carriage rates of *S. pneumoniae*, vaccinated children would not be exposed to numerous pneumococcal protein antigens.[23] This, in turn, might have reduced the production of antipneumococcal antibodies in this subgroup of children. Indeed, it has already been demonstrated that children frequently colonized with *S. pneumoniae* present higher levels of antiprotein antibodies compared with those in which colonization was not found.[24]

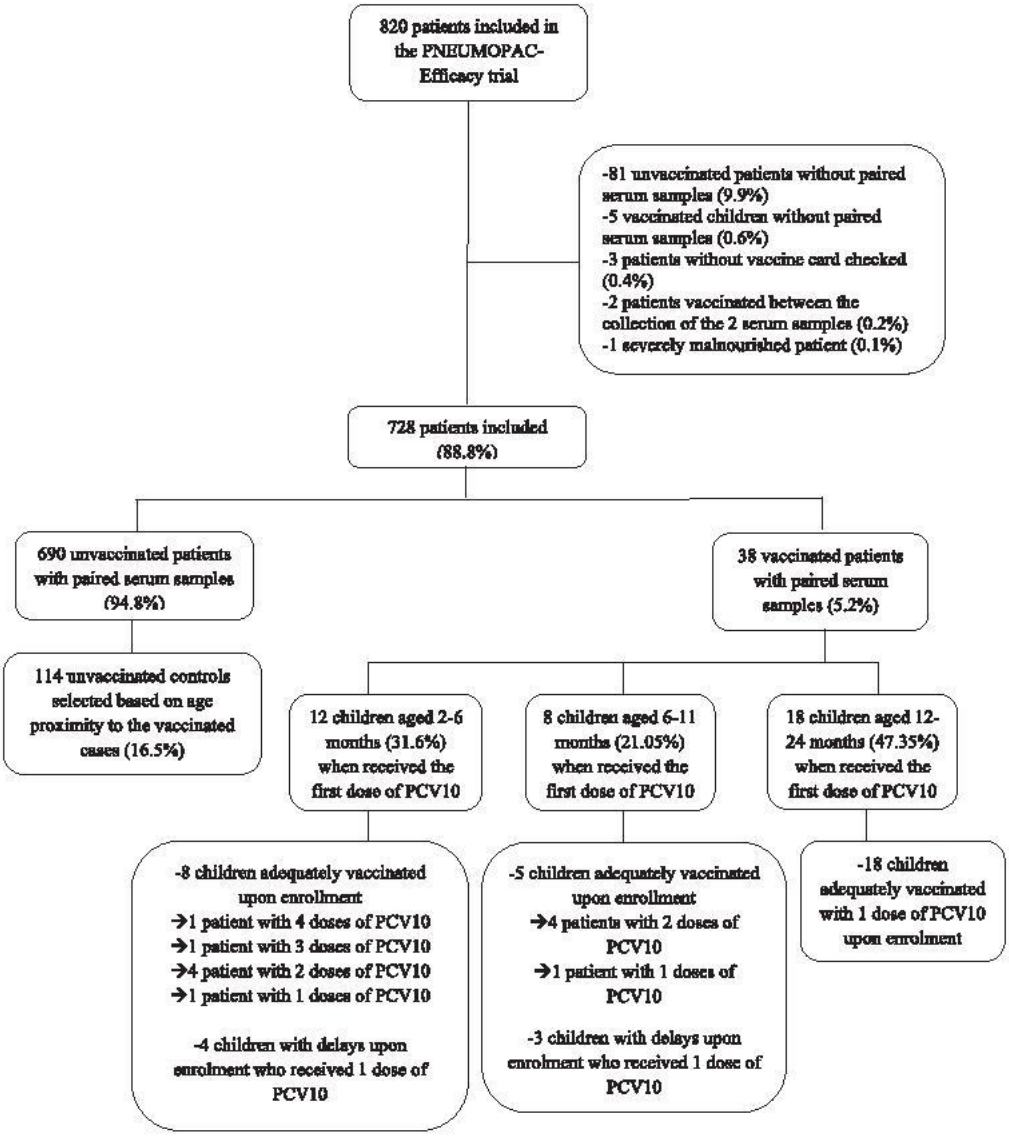

FIGURE 1. Flow chart of the included and excluded cases of this study and description of the vaccination status and the number of doses of PCV administered to each vaccinated child.

TABLE 2. Comparison of the Antibody Levels of the First Serum Sample Taken Upon Enrollment With a Dilution Factor of 1:1600 Between Vaccinated (Cases) and Unvaccinated (Controls) Children

	MFI Values (Median [25th–75th percentile])		
Protein	Vaccinated (n=38)	Unvaccinated (n=114)	*P*
Ply	57 (35.5–113.75)	74 (43.5–189)	0.048
CbpA	1224 (124–3707)	2064 (242.75–5832.75)	0.171
PspA*	112 (53.5–266)	191 (84.25–594.75)	0.018
PcpA	363 (38.5–1010.5)	637.5 (69.5–1380.75)	0.074
PhtD	435 (80–1034)	735.5 (104.75–2350)	0.089
StkP	101 (30–139.75)	162 (47.75–594)	0.001
PcsB	443 (64–1677.25)	1212.5 (104.5–3380.5)	0.028
Haemophilus influenzae	130 (56.75–207.75)	110.5 (79.5–196.5)	0.670
Moraxella catarrhalis	149.5 (104–303.75)	111 (76–188.25)	0.015

*PspA 1 and 2 were conjugated on the same bead set.

CbpA indicates choline-binding protein A; PcpA, pneumococcal choline-binding protein A; PcsB, protein required for cell wall separation of group B streptococcus; PhtD, pneumococcal histidine triad protein D; Ply, pneumolysin; StkP, serine/threonine protein kinase.

TABLE 3. Comparison of the Frequency of Antibody Response Detection Between Vaccinated (Cases) and Unvaccinated (Controls) Children With CAP

Protein	Frequency of Antibody Increase (n [%]) Vaccinated (N=38)	Unvaccinated (N=114)	*P*
Ply	0 (0%)	3 (2.6%)	0.573
CbpA	2 (5.3%)	3 (2.6%)	0.599
PspA*	0 (0%)	2 (1.8%)	1
PcpA	1 (2.6%)	9 (7.9%)	0.453
PhtD	0 (0%)	6 (5.3%)	0.337
StkP	2 (5.3%)	6 (5.3%)	1
PcsB	1 (2.6%)	6 (5.3%)	0.681
Streptococcus pneumoniae†	4 (10.5%)	17 (14.9%)	0.497
Haemophilus influenzae	4 (10.5%)	12 (10.5%)	1
Moraxella catarrhalis	4 (10.5%)	4 (3.5%)	0.108
Responses to multiple pathogens			
S. pneumoniae and *H. influenzae*	2 (5.3%)	1 (0.9%)	0.154
S. pneumoniae and *M. catarrhalis*	0 (0%)	2 (1.8%)	1
H. influenzae and *M. catarrhalis*	0 (0%)	0 (0%)	–
S. pneumoniae, *H. influenzae* and *M. catarrhalis*	0 (0%)	0 (0%)	–
Overall detection rate‡	10 (26.3%)	29 (25.4%)	1

*PspA 1 and 2 were conjugated on the same bead set.

†Frequency of antibody response detection to at least one pneumococcal protein.

‡Detection of responses against any of the studied pathogens.

CbpA indicates choline-binding protein A; PcpA, pneumococcal choline-binding protein A; PcsB, protein required for cell wall separation of group B streptococcus; PhtD, pneumococcal histidine triad protein D; Ply, pneumolysin; StkP, serine/threonine protein kinase.

TABLE 4. Effect of the Time Interval Between the Date of Administration of the First and/or Last Dose of PCV10 and the Date of Collection of the First Serum Sample on the Levels of Natural Antibodies Against Protein Antigens

Antibody Concentration in the First Serum Sample Against	Time Interval Between the First Dose of PCV and Collection of the First Sample *r**	*P**	Time Interval Between the Last Dose of PCV and Collection of the First Sample *r**	*P**
Ply	0.041	0.805	0.122	0.467
CbpA	−0.158	0.344	−0.044	0.793
PspA†	−0.099	0.555	0.067	0.688
PcpA	0.036	0.829	0.017	0.921
PhtD	−0.085	0.612	−0.038	0.819
StkP	−0.174	0.297	0.036	0.829
PcsB	−0.112	0.505	−0.066	0.695
Haemophilus influenzae	0.384	0.017	0.151	0.367
Moraxella catarrhalis	0.124	0.460	0.243	0.141

*Similar results were found when using a 1:400 dilution factor (data not shown).

†PspA 1 and 2 were conjugated on the same bead set.

CbpA indicates choline-binding protein A; PcpA, pneumococcal choline-binding protein A; PcsB, protein required for cell wall separation of group B streptococcus; PhtD, pneumococcal histidine triad protein D; Ply, pneumolysin; *r*, correlation coefficient; StkP, serine/threonine protein kinase.

Despite the differences of the antibody levels, no statistically significant difference between vaccinated and unvaccinated children was found regarding the frequency of antibody responders against the studied pneumococcal proteins upon contraction of CAP. A possible explanation for this finding is that in vaccinated children, disease was caused by nonvaccine serotypes. Increases in the rate of invasive pneumococcal disease caused by nonvaccine serotypes have been reported after the introduction of PCVs.[25] The antigens used in this study are, however, highly conserved and almost universally present in all strains of *S. pneumoniae*.[23,26–31] Therefore, the antibody response against them should not be substantially affected by the serotype of pneumococcus causing CAP. In addition, high levels of several antiprotein pneumococcal antibodies reduce adherence of the pneumococcus to human lung epithelial cells[32] and have been associated with a reduced frequency of ARIs in children.[10,13] Therefore, although children vaccinated with PCV10 were protected against disease caused by the vaccine-covered serotypes, they lacked the protective effect of high levels of antiprotein antibodies, rendering them sensitive to nonvaccine serotypes. This fact, in turn, might also have contributed to the similar frequency of antibody response found for vaccinated and unvaccinated children. However, it is important to recall that this study evaluated children with nonsevere pneumonia treated as outpatients, in whom viruses have been demonstrated to be the major cause of CAP instead of bacteria.[33,34] Therefore, a lower impact of pneumococcal vaccination is expected in this group of patients. AQ5

No difference was found either for the natural antibody levels or the frequency of antibody responders against *H. influenzae* associated with CAP between vaccinated and unvaccinated children. On the other hand, vaccinated children presented higher levels of antibodies against *M. catarrhalis* when compared with unvaccinated children. One possible explanation is that vaccinated children might have had a greater carriage rate for this bacterium as colonization by the common vaccine serotypes of *S. pneumoniae* was prevented and possibly allowed a higher rate of colonization by *M. catarrhalis*. Accordingly, changes in the rate of nasopharyngeal colonization by *M. catarrhalis* have already been reported in certain circumstances known to affect the local flora, such as day-care attendance.[35] However, most studies evaluating the rates of colonization by *M. catarrhalis* after pneumococcal vaccination found no difference between vaccinated and unvaccinated children.[36,37]

The limitations of our study must be acknowledged. First, the group of children who received PCV10 was small and heterogeneous regarding the completion of the vaccination scheme and the number of PCV10 doses that were administered. Nevertheless, we found no effect for the number of doses of PCV10 or for the time interval between the first/last dose of vaccine and first serum sample collection on either the levels of natural antibodies or the frequency of antibody responses in this group of children. This, in turn, might suggest that although the group of vaccinated children was indeed heterogeneous, the differences of the vaccination schemes did not significantly affect the outcome variables from this study. Also, the groups of vaccinated children and their respective unvaccinated controls were very similar with regard to age and other clinical features, so that the differences found herein cannot be explained by these factors. It is important to recall that this was an exploratory study, and our findings should be confirmed in studies with a larger and homogeneous sample regarding vaccination status. Second, we had no data from the colonization status from the evaluated children or definitive tests on the etiology of CAP. Therefore, the association between the findings from this study and putative changes in the rates of colonization or etiology of CAP are only theoretical. Finally, we had no data on the use of other vaccines which might have affected the rates of colonization/infection by the studied pathogens, such as the *H. influenzae* type b vaccine. Nevertheless, it has already been demonstrated that the coverage of the *H. influenzae* type b vaccine is high in the pediatric population in Brazil, so it probably did not affect the results from this study.[38]

In conclusion, this study demonstrated that children who were vaccinated with PCV10 had lower levels of natural antibodies against 4 protein antigens from *S. pneumoniae* when compared with unvaccinated children with similar age and clinical presentation. This could reflect lower *S. pneumoniae* carriage rates in the vaccinated children as a consequence of PCV10 vaccination. Despite the differences in the levels of natural antibodies, no difference was found in the rates of antibody responders associated with CAP against protein antigens from *S. pneumoniae, H. influenzae* and *M. catarrhalis* among vaccinated and unvaccinated children with CAP.

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X. DISCUSSÃO

1. Validação de um teste sorológico em multiplex com antígenos protéicos de *S. pneumoniae*

Nossos achados sugerem que a quantificação de IgG contra proteínas pneumocócicas em amostras pareadas de soro apresenta boa acurácia para o diagnóstico de doença pneumocócica invasiva. Um aumento ≥2 vezes no nível basal de anticorpos é um ponto de corte adequado para a maior parte dos antígenos avaliados. No entanto, o uso de diferentes pontos de corte para o aumento no nível de anticorpos pode melhorar a precisão do teste para alguns antígenos, especialmente para PcpA.

O papel da sorologia no diagnóstico de infecção pneumocócica tem sido amplamente discutido nas últimas décadas (Korppi et al., 2008). *S. pneumoniae* freqüentemente coloniza o trato nasofaríngeo de crianças (Bogaert et al., 2004), e essa forma de contato com pneumococo pode promover um aumento no nível de anticorpos contra antígenos desta bactéria (Prevaes et al., 2012). No entanto, Turner et al. demonstraram que a aquisição de uma nova colonização pelo *S. pneumoniae* não apresentou associação estatisticamente significante com um aumento ≥2 vezes nos níveis de anticorpos para 27 proteínas pneumocócicas (Turner et al., 2013). No nosso estudo, demonstramos também que a resposta sorológica a antígenos protéicos de *S. pneumoniae* foi altamente específica ao comparar crianças com doença pneumocócica invasiva (DPI) e os controles negativos não colonizados. Desse modo, embora a necessidade de amostras pareadas represente um obstáculo para o uso de sorologia na prática clínica, ela ainda é uma opção viável para estudos epidemiológicos. Finalmente, as técnicas sorológicas estão em constante desenvolvimento e superaram limitações prévias como o número e tipo de antígenos passíveis de serem incluídos em cada teste (Andrade et al., 2014).

Diversas proteínas pneumocócicas foram descritas na última década e seu uso em testes sorológicos pode ter vantagens em comparação com os polissacarídeos capsulares comumente utilizados, sobretudo se a informação do sorotipo infectante não for necessária. Por exemplo, Olaya-Abril et al descreveram recentemente uma novo teste usando 95 proteínas pneumocócicas recombinantes que haviam sido originalmente descritas por meio de técnicas de proteômica, representando a versatilidade de antígenos protéicos para uso em diagnóstico sorológico (Olaya-Abril et al., 2017). Todos os antígenos que incluímos em nosso teste sorológico são descritos na literatura como altamente específicos para *S. pneumoniae* e amplamente distribuídos entre suas cepas (Tai, 2006; Giefing et al., 2008; van der Poll & Otal, 2009). Portanto, o uso de proteínas pneumocócicas permite a identificação de respostas sorológicas contra *S. pneumoniae* independentemente do sorotipo da cepa infectante. Além disso, o desenvolvimento de técnicas em multiplex permitiu a avaliação de resposta a múltiplos antígenos na mesma reação (Andrade, 2014), representando uma opção mais econômica para melhorar a sensibilidade do teste através da combinação de diferentes antígenos. Testes sorológicos incluindo até 64 antígenos pneumocócicos já foram descritos e demonstraram-se robustos e sem efeitos deletérios pelo uso em multiplex, representando uma ferramenta engenhosa para a quantificação de IgG a proteínas pneumocócicas (Jiménez-Munguía et al., 2015).

As diferenças encontradas para os níveis de anticorpos nos soros de fase aguda podem ser explicadas pela diferença de idade entre os grupos de estudo. A produção de anticorpos contra antígenos protéicos de *S. pneumoniae* começa em diferentes idades para cada antígeno (Rapola et al., 2000; Holmlund et al., 2006; Zhang et al., 2006; Holmlund et al., 2009; Simell et al., 2009; Lebon et al., 2011; Prevaes et al., 2012; Hagerman et al., 2013) e, portanto, a idade das crianças influencia diretamente o nível de anticorpos gerados contra esses antígenos. No entanto, é importante enfatizar que os níveis de anticorpos no soro de fase

aguda não afetaram a associação entre o grupo de estudo e a detecção de resposta sorológica contra PspA, PcpA, PhtD e PscB (Tabela 4). Não foi encontrada associação independente entre o grupo de estudo e a detecção de resposta sorológica para Ply, CbpA e StkP, e é possível que isso tenha sido devido ao pequeno tamanho da amostra neste estudo.

Os pontos de corte ideais para o aumento nos níveis de anticorpos contra cada antígeno definidos neste estudo foram baseados na maior acurácia para o diagnóstico de infecção pneumocócica. A acurácia destes pontos de corte foi, em geral, similar ao do ponto de corte ≥2 vezes. No entanto, o ponto de corte ≥1,52 vezes para um aumento nos níveis de anticorpos basais contra PcpA demonstrou-se mais preciso em comparação com um ponto de corte ≥2 vezes para um aumento nos níveis de anticorpos contra o mesmo antígeno (88,89% vs. 77,78%). Um aumento discreto na acurácia do teste foi identificado ao utilizar um ponto de corte ≥3,16 vezes para um aumento nos anticorpos contra Ply e de um ponto de corte ≥1,97 vezes para um aumento nos anticorpos contra PspA em comparação com o ponto de corte ≥2 vezes contra esses mesmos antígenos (72,22% *vs.* 69,44% e 77,78% *vs.* 75%, respectivamente). Portanto, o uso de um aumento ≥2 vezes nos níveis de anticorpos basais como ponto de corte pode ser um critério de diagnóstico útil para a detecção de infecção pneumocócica quando utilizando os antígenos protéicos investigados neste estudo; o uso de pontos de cortes distintos, no entanto, pode melhorar ainda mais a acurácia do teste, particularmente para anticorpos contra PcpA. O uso de um aumento ≥2 vezes nos níveis de anticorpos já havia sido validado para Ply, através da quantificação dos níveis de anticorpos em crianças finlandesas saudáveis (Nohynek et al., 1995), o que contrasta com o ponto de corte mais alto encontrado para este antígeno no presente estudo. No entanto, ao comparar a especificidade do ponto de corte ideal selecionado aqui com a do ponto de corte ≥2 vezes (100% vs. 95.65%, respectivamente), observa-se apenas um pequeno aumento na especificidade do teste ao utilizar o ponto de corte ≥ 3.16 vezes. Portanto, o uso de um ponto

de corte ≥2 vezes para um aumento nos níveis de anticorpos basais ainda representa uma opção razoável para o diagnóstico de pneumonia pneumocócica usando o antígeno pneumolisina.

O uso de combinações de antígenos protéicos pneumocócicos representa uma estratégia interessante para melhorar a sensibilidade de um teste sorológico. Mostramos neste estudo que, ao utilizar todos os antígenos incluídos, a detecção de resposta sorológica contra pelo menos um antígeno é altamente sensível e específica para a presença de infecção pneumocócica. É importante ressaltar que, entre os antígenos estudados, PcpA e PcsB-N foram os antígenos com maior acurácia, e sua inclusão deve ser considerada ao desenvolver um teste sorológico contra *S. pneumoniae*. Posfay-Barbe et al. mostraram que o uso de múltiplos antígenos melhora significativamente a sensibilidade da detecção da resposta sorológica contra *S. pneumoniae*, particularmente se PcpA foi incluído na combinação (Posfay-Barbe et al., 2011). Além disso, Jiménez-Munguía et al. também descreveram que os níveis de IgG contra PcpA foram três vezes menores em soros de crianças com pneumonia coletados até 10 dias de doença, quando comparadas aos níveis de IgG dos controles negativos (Jiménez-Munguía et al., 2015). Resultados semelhantes foram encontrados para a avaliação das respostas sorológicas em uma coorte de 690 crianças com pneumonia não-grave adquirida na comunidade, na qual PcpA e PcsB foram os antígenos com a maior taxa de detecção de resposta sorológica (Borges et al., 2015).

As limitações desta investigação devem ser comentadas. Em primeiro lugar, houve diferenças importantes entre controles positivos e negativos, isto é, nacionalidade, idade, vacinação e diferentes níveis de anticorpos nas amostras de soro à admissão. No entanto, demonstramos que essas diferenças não afetaram a detecção de resposta sorológica a pelo menos quatro proteínas pneumocócicas, incluindo PcpA e PcsB-N. Além disso, um estudo recente demonstrou que a vacinação com PCV10 não afetou a frequência de detecção de

resposta sorológica em uma coorte de crianças com PAC não grave (Andrade et al., 2016). Em segundo lugar, os grupos de controle positivo e negativo incluídos neste estudo tiveram poucos participantes. É possível que o pequeno número de participantes neste estudo tenha comprometido a associação independente entre o grupo de estudo e a detecção de resposta sorológica contra Ply, CbpA e StkP. O pequeno número de pacientes incluídos é devido à dificuldade em adquirir amostras de soro pareadas de pacientes pediátricos. Além disso, menos de 5% dos casos de PAC pediátrica desenvolve bacteremia (Shah et al., 2010). Os controles negativos eram crianças com faringite, que não são crianças estritamente saudáveis. No entanto, é importante lembrar que a faringite em crianças menores de 5 anos é predominantemente causada por vírus, e *S. pneumoniae* não é reconhecido como agente etiológico nesta situação (Hseh et al., 2011). É possível que as respostas sorológicas contra *S. pneumoniae* detectadas nos dois pacientes com faringite tenham sido causadas por uma resposta policlonal provocada pelo EBV (Freijd & Rosen, 1984) ou por uma reação cruzada com anticorpos reativos contra o Streptococcus do Grupo A. Além disso, como a colonização não foi avaliada novamente durante coleta das segundas amostras de soro no grupo de controles negativos, é possível que a aquisição de uma nova cepa de *S. pneumoniae* tenha ocorrido entre a coleta as amostras de soro e gerado uma resposta sorológica. Finalmente, a avaliação da colonização por *S. pneumoniae* via esfregaço orofaríngeo não é o método mais sensível para detecção desta bactéria (Satzke et al., 2013).

Em conclusão, demonstramos que a sorologia usando múltiplas proteínas pneumocócicas é um método promissor para o diagnóstico de infecção pneumocócica em crianças com pneumonia. Embora o ponto de corte ≥2 vezes para um aumento nos níveis basais de anticorpos seja adequado para a maioria dos antígenos, diferentes cortes podem ser mais acurados para alguns antígenos, como aumento ≥1,52 vezes nos níveis de anticorpos basais contra PcpA. Além disso, quando se utilizam os antígenos aqui estudados, a detecção

de resposta de anticorpos contra pelo menos um antígeno é altamente sensível e específica para o diagnóstico de infecção por *S. pneumoniae*.

2. Desenvolvimento e validação de um teste de avidez em multiplex com antígenos protéicos de *S. pneumoniae*

Descrevemos neste estudo o desenvolvimento e a validação de um teste de avidez em multiplex utilizando proteínas pneumocócicas. Quando o teste foi aplicado a um cenário clínico, descobrimos que crianças com infecção pneumocócica apresentam menor avidez média de IgG contra antígenos protéicos quando comparados com crianças sem evidência de infecção pneumocócica. Além disso, a análise da curva ROC sugere que estudos de avidez podem ser úteis como ferramenta diagnóstica.

A avidez de IgG contra os polissacarídeos de *S. pneumoniae* tem sido amplamente estudada em ensaios clínicos avaliando vacinas pneumocócicas, como ferramenta para avaliar a qualidade da resposta de anticorpos (Ekström et al., 2007; Ekström et al., 2013). Os testes de avidez podem ser utilizados como uma medida da funcionalidade da resposta de anticorpos e uma correlação negativa entre a avidez do anticorpo e a concentração de anticorpos necessária para a atividade opsonofagocítica/bactericida já foi relatada em estudos anteriores (Schlesinger & Granoff, 1992; Antilla et al., 1999; Usinger & Lucas, 1999). O uso de avidez para avaliar a resposta de anticorpos contra proteínas pneumocócicas, no entanto, só foi feito em poucos estudos, principalmente testes de vacinas experimentais utilizando modelos animais (Chen et al., 2015; Olasdottir et al., 2012). Portanto, no cenário atual onde novas vacinas baseadas em antígenos protéicos estão em desenvolvimento (Brooks et al., 2015), a validação de protocolos econômicos para avaliar a avidez de IgG contra estes antígenos é necessária. Neste estudo, descrevemos a validação de um teste de avidez robusto,

no qual não houve efeito deletério devido ao multiplex. No entanto, as leituras de intensidade de fluorescência (que são proporcionais às concentrações de anticorpos IgG na amostra) neste teste de avidez devem permanecer dentro de um intervalo predeterminado (entre 100 e 7000 IMF) para garantir a consistência dos resultados. Além disso, múltiplas diluições da mesma amostra podem ser necessárias, uma vez que as concentrações de anticorpos para diferentes antígenos numa mesma amostra podem variar.

Aqui, descobrimos que as crianças com doença pneumocócica apresentam menor avidez de anticorpos contra maior parte dos antígenos protéicos avaliados tanto em amostras de fase aguda quanto na convalescença, em comparação com crianças sem doença pneumocócica. Resultados semelhantes foram encontrados ao comparar apenas crianças com DPI com controles saudáveis. Até o momento, a avidez da IgG contra antígenos protéicos de *S. pneumoniae* só foi avaliada clinicamente uma vez, em um estudo que avaliou a avidez da IgG contra Ply, CbpA e PspA em um grupo composto por 20 crianças com DPI e 20 controles saudáveis (Ota et al., 2011). Nesse estudo, crianças com DPI apresentaram maior avidez de IgG na convalescença da doença, quando comparadas ao grupo controle. Etudos anteriores que avaliaram a avidez dos anticorpos contra polissacarídeos capsulares, no entanto, também encontraram menor avidez em crianças com doença pneumocócica. Por exemplo, crianças com infecções respiratórias recorrentes apresentaram menor avidez de IgG contra polissacarídeos capsulares em comparação com controles saudáveis, em um estudo avaliando os níveis de anticorpos contra 12 sorotipos pneumocócicos (Fried et al., 2013). Baixas avidez e atividade opsonofagocítica também foram relatadas contra o sorotipo infectante em crianças com DPI (Oishi et al., 2013). Neste cenário, a presença de anticorpos de alta avidez contra polissacarídeos pneumocócicos tem sido descrita como um fator protetor contra a infecção pneumocócica (Antilla et al., 1999; Usinger & Lucas, 1999; Musher et al., 2000). É possível, portanto, que a maior avidez contra as proteínas

pneumocócicas no grupo de crianças sem doença pneumocócica aqui encontrada também possa ser um determinante de proteção contra infecção por esta bactéria.

Neste estudo, não encontramos um aumento estatisticamente significante na avidez de IgG entre amostras de fase aguda e convalescença. Um aumento da avidez contra proteínas pneumocócicas foi reportado por Ota *et al*, 2011, que encontrou menor avidez de IgG contra Ply, CbpA e PspA na fase aguda da doença em crianças com DPI em comparação com a fase de convalescença (Ota et al., 2011). Estas diferenças, no entanto, também não foram estatisticamente significantes. É possível que o pequeno tamanho amostral do nosso estudo e do trabalho de Ota et al. tenha comprometido a detecção de aumentos de avidez entre amostras de fase aguda e convalescença, os quais representariam a maturação dos anticorpos antígeno-específicos em resposta à exposição às proteínas pneumocócicas.

Na avaliação da avidez dos anticorpos como ferramenta diagnóstica para a doença pneumocócica, descobrimos que diferentes proteínas tem poder discriminativo variável para a detecção de infecção por *S. pneumoniae*. Os anticorpos contra StkP-C e PcpA apresentaram alta precisão e podem ser considerados candidatos para um teste de avidez na prática clínica. O uso da avidez tem a vantagem de exigir apenas uma amostra de soro para fornecer informações diagnósticas, em comparação com ensaios sorológicos convencionais que requerem amostras pareadas. Por exemplo, a avidez de IgG contra polissacarídeos pneumocócicos foi avaliada para fins diagnósticos por Fried et al, 2013, que descreveram um alto poder discriminativo da avidez para distinguir entre grupos de crianças com infecções respiratórias bacterianas recorrentes e controles saudáveis (Fried et al., 2013). No entanto, a validação adequada de protocolos de avidez e a definição de pontos de corte para o diagnóstico de doença aguda ainda são necessárias. Neste, apresentamos uma avaliação preliminar dos pontos de corte ideais para o diagnóstico de doença pneumocócica, e encontramos uma alta variabilidade dos pontos de corte de avidez ideiais para cada proteína

escolhidos com base na melhor acurácia do teste. Esse achado reforça a necessidade de uma validação individualizada para cada antígeno protéico.

As limitações desta investigação devem ser observadas. Em primeiro lugar, houve diferenças importantes no grupo de crianças sem doença pneumocócica (ou seja, entre o subgrupo de crianças saudáveis e o subgrupo de crianças com PAC sem resposta sorológica a *S. pneumoniae*), como nacionalidade, idade e intervalo de amostragem. No entanto, quando a avidez de IgG entre os subgrupos foi avaliada, a diferença foi encontrada apenas para PhtD. É possível que a idade mais avançada no subgrupo de crianças saudáveis tenha contribuído para a maior avidez encontrada contra PhtD devido ao maior tempo de possível exposição a *S. pneumoniae*. Em segundo lugar, uma quantidade considerável de amostras foram excluídas da análise devido à detecção de níveis de anticorpos fora do intervalo de fluoresc6encia predeterminado. Infelizmente, não foram realizadas repetições para essas amostras devido a restrições de material e tempo. No entanto, é importante enfatizar que este foi um estudo preliminar com o objetivo de padronizar e aplicar o primeiro teste de avidez em multiplex contra oito antígenos pneumocócicos, e o protocolo descrito deve ser avaliado novamente usando um maior tamanho amostral. Nós incluímos 5 crianças com menos de 6 meses de idade, e que poderiam, portanto, ainda estar sob a proteção de anticorpos maternos. É importante ressaltar, no entanto, que aumentos nos níveis de anticorpos contra proteínas pneumocócicas já foram relatados em crianças com menos de 6 meses de idade apresentando infecções sintomáticas, como PAC e OMA (Andrade et al, 2016a; Andrade et al, 2016b; rapola et al, 2001). Portanto, visto que crianças com idade inferior a 6 meses podem produzir respostas sorológicas quantitativas apesar da presença de anticorpos maternos, nós hipotetizamos que a avidez de anticorpos contra proteínas pneumocócicas também deva ser afetada durante um episódio infeccioso. Nós encontramos um decréscimo nos níveis de fluorecência para PcpA aos o tratamento com tiocianeto de sódio na concentração 6M. No

entanto, ainda assim obtivemos uma alta correlação entre os níveis de fluorescência de beads conjugadas com esta proteínas que foram submetidas ou não a um pré-tratamento com o agente caotrópico (Figura 2). Finalmente, não tivemos dados sobre colonização por *S. pneumoniae* para as crianças incluídas e como essa forma de contato com o pneumococo pode afetar a avidez da IgG anti-proteína. Foi relatado que crianças que mantém contato com outras crianças, um fator de risco reconhecido para a colonização pneumocócica, apresentaram maior avidez de IgG contra alguns sorotipos pneumocócicos do que crianças sem interação com outras crianças (Salt et al., 2007). Deste modo, o efeito da colonização por *S. pneumoniae* sobre a avidez dos anticorpos contra proteínas pneumocócicas deve ser o foco de estudos futuros.

Em conclusão, este foi o primeiro relato do desenvolvimento e validação de um teste de avidez em multiplex usando proteínas pneumocócicas, o qual foi robusto e não apresentou efeito deletério do multiplex. Quando aplicado a um cenário clínico, o teste descrito foi capaz de identificar diferenças na avidez da IgG contra antígenos protéicos entre grupos de crianças com e sem doença pneumocócica, com menor avidez encontrada no grupo de crianças com doença pneumocócica. A avidez de anticorpos contra proteínas pneumocócicas também pode ser utilizada como ferramenta diagnóstica para infecção pneumocócica, sendo que os antígenos StkP-C e PcpA devem ser considerados para inclusão em tal teste.

3. Papel do raio-X de tórax no diagnóstico de CAP

Esta investigação demonstrou que crianças com pneumonia radiologicamente confirmada apresentam maior freqüência de infecção por *S. pneumoniae* do que crianças com raio-X de tórax normal. A presença de infecção pelo pneumococo foi associada independentemente à pneumonia radiologicamente confirmada entre crianças com diagnóstico clínico de PAC tratadas ambulatorialmente. Além disso, a presença de um raio-X

de tórax normal teve um alto valor preditivo negativo para a detecção de resposta sorológica contra *S. pneumoniae*.

Encontramos uma maior freqüência de resposta sorológica contra vários antígenos de *S. pneumoniae* no grupo de crianças com pneumonia radiologicamente confirmada quando comparado ao de crianças com raio-X de tórax normal. Esse achado corrobora os resultados de estudos anteriores que demonstraram que a presença de infiltrados alveolares em radiografias de tórax está associada a pneumonia bacteriana (Virkki et al., 2002). Nascimento-Carvalho et al (2014) também relataram que a infecção por *S. pneumoniae* foi detectada mais frequentemente entre crianças hospitalizadas com PAC que apresentaram achados radiográficos de pneumonia do que em crianças com raio-X de tórax normal (Nascimento-Carvalho et al., 2014). Por outro lado, crianças com raio-X de tórax normal apresentaram maior incidência de infecção viral (Nascimento-Carvalho et al., 2014). Este é o primeiro estudo da associação entre infecção pneumocócica e pneumonia radiologicamente confirmada entre crianças não hospitalizadas com diagnóstico clínico de PAC.

Conseqüentemente, o valor preditivo negativo do raio-X de tórax normal para detecção de infecção pneumocócica foi alto (86,3% [IC 95%: 82,4% -89,7%]). Embora uma associação entre infecção bacteriana e infiltrado alveolar/consolidação tenha sido previamente descrita (Virkki et al., 2002), esses achados não podem estabelecer de forma confiável o diagnóstico etiológico de PAC (Don, 2009; Xavier-Souza, 2013). Portanto, nosso achado de que o raio-X de tórax normal tem um alto valor preditivo negativo para a infecção pneumocócica pode ajudar na interpretação deste exame. Encontramos um alto valor preditivo negativo para o raio-X de tórax normal em uma população com alta prevalência de infecção pneumocócica (Nascimento-Carvalho et al., 2008), reforçando assim nossos resultados. Em conjunto, nossos dados mostram que as crianças com PAC não grave com pneumonia radiologicamente confirmada têm maior chance de infecção por *S. pneumoniae*,

enquanto crianças com um raio-X de tórax normal provavelmente não apresentam infecção por este agente e podem não se beneficiar do uso empírico de antibióticos.

Dados de ensaios clínicos avaliando vacinas pneumocócicas reforçam a relação entre infecção pneumocócica e pneumonia radiologicamente confirmada, já que um efeito diferencial da vacinação pneumocócica foi encontrado nas taxas de PAC pediátrica de acordo com os critérios diagnósticos aplicados. Por exemplo, a eficácia da vacina PCV 10-valente foi significativamente maior para crianças com consolidação no raio-X de tórax do que para crianças com infiltrados alveolares ou apenas com diagnóstico clínico de PAC (Tregnaghi et al., 2014). O maior impacto da vacinação pneumocócica em crianças com consolidação no raio-X de tórax sugere que pacientes com este diagnóstico radiológico apresentam maior incidência de infecção pneumocócica. Esses achados são consistentes com os relatados por Lucero et al, 2009, que demonstraram uma boa eficácia da vacina do PCV 11-valente para crianças com pneumonia radiologicamente confirmada (definida como presença de consolidação) e eficácia vacinal mínima para crianças com apenas diagnóstico clínico de pneumonia (Lucero et al., 2009). Esses ensaios clínicos fornecem evidências indiretas sobre a etiologia da pneumonia em crianças com padrões radiológicos distintos, indicando que crianças com pneumonia radiologicamente confirmada apresentam uma maior freqüência de infecção por *S. pneumoniae*.

O papel do raio-X de tórax no manejo de crianças com PAC, no entanto, tem sido amplamente discutido. Bradley et al (2011), por exemplo, recomendam que a radiografia de tórax só seja realizada em crianças hospitalizadas ou com hipoxemia, dificuldade respiratória significativa, suspeita de complicações ou falência terapêutica (Bradley et al., 2011). Esta posição é corroborada por Harris et al (2011), que afirmaram que as crianças com sintomas e sinais sugestivos de PAC e tratadas ambulatorialmente não devem ser submetidas a um raio-X de tórax rotineiramente (Harris et al., 2011). Essas recomendações devem-se em parte a

estudos anteriores que demonstraram que a pneumonia bacteriana não pode ser diferenciada com segurança da pneumonia não bacteriana com base apenas nos achados de um raio-X de tórax anormal (Toikka et al., 2000; Korppi et al., 2008; Don et al., 2009). Além disso, evidências atuais sugerem que a realização de um raio-X de tórax não melhora o desfecho clínico de pacientes pediátricos com PAC (Swingler et al., 1998). É importante enfatizar, no entanto, que quando o impacto do raio-X de tórax sobre o manejo de crianças com PAC foi avaliado, os pacientes receberam antibióticos a critério do médico assistente independentemente dos achados radiológicos, limitando assim o potencial benefício de um estudo radiológico como ferramenta diagnóstica com implicações terapêuticas (Swingler et al., 1998). De acordo com estes achados, Harris et al (2011) recomendam o uso de antibióticos para todas as crianças com diagnóstico claro de PAC (Harris et al., 2011). Ambas as diretrizes concordam, no entanto, que as crianças pequenas não exigem o uso rotineiro de antibióticos, já que a maioria apresentará infecção viral (Bradley et al., 2011; Harris et al., 2011). Nesse cenário, embora a radiografia de tórax não seja capaz de distinguir inequivocamente os agentes etiológicos da PAC, ela pode ajudar a diferenciar diferentes padrões de infecção respiratória inferior. Evidências recentes demonstraram diferenças importantes entre crianças com ou sem pneumonia radiologicamente confirmada tanto na apresentação quanto na evolução clínica. As crianças com pneumonia radiologicamente confirmada têm maior freqüência e maior persistência de febre (Key et al., 2008; Cardoso et al., 2011; Fontoura et al., 2012) e também evoluem de forma mais grave, com internação mais longa, maior necessidade de suporte respiratório e maiores taxas de falha terapêutica (Kelly et al., 2016). Essas diferenças indicam que crianças com e sem pneumonia radiologicamente confirmada podem ter padrões diferentes de infecção do trato respiratório inferior, e o raio-X de tórax, quando realizado, pode ajudar a conduzir casos duvidosos de PAC não grave.

Também descobrimos que crianças com pneumonia radiologicamente confirmada apresentaram níveis mais altos de anticorpos contra várias proteínas pneumocócicas tanto na admissão quanto na convalescença. Foi relatado anteriormente que níveis mais baixos de anticorpos anti-pneumocócicos na admissão estão associados a uma maior freqüência de resposta sorológica contra *S. pneumoniae* devido a particularidades técnicas (Borges et al., 2015). Portanto, o nível de anticorpos na admissão dos pacientes incluídos no nosso estudo provavelmente não foi responsável pela maior taxa de resposta sorológica contra o pneumococo em crianças com pneumonia radiologicamente confirmada. O nível mais elevado de anticorpos na admissão neste grupo de crianças, por sua vez, pode ter sido causado por colonização prévia por *S. pneumoniae*. A colonização nasofaríngea é reconhecidamente parte da história natural da doença pneumocócica invasiva, que ocorre se as barreiras imunológicas são atravessadas pelas bactérias colonizadoras (Bogaert et al., 2004). Além disso, as crianças com diagnóstico clínico e radiológico de pneumonia também são mais freqüentemente colonizadas pelo *S. pneumoniae* quando comparadas aos controles saudáveis (Chappuy et al., 2013). Portanto, é possível que uma maior taxa de colonização por *S. pneumoniae* em crianças com pneumonia radiologicamente confirmada tenha provocado os níveis mais altos de anticorpos anti-pneumocócicos presentes neste subgrupo.

Não encontramos diferença nas taxas de resposta sorológica contra *H. influenzae* e *M. catarrhalis* neste estudo, possivelmente devido ao baixo número de respondedores em nosso grupo de estudo. Contudo, foram encontrados níveis discretamente superiores de anticorpos contra *H. influenzae* para crianças com pneumonia radiologicamente confirmada, bem como níveis mais baixos de anticorpos contra *M. catarrhalis*. Sabe-se que vários agentes bacterianos competem para colonizar o trato nasofaríngeo de pacientes pediátricos, criando um processo dinâmico de “turnover” de agentes colonizadores (Chappuy et al., 2013). Um aumento das taxas de colonização por *S. pneumoniae* também pode ter contribuído para

reduzir os níveis de anticorpos contra *M. catarrhalis* nas amostras de crianças com pneumonia radiologicamente confirmada na admissão. Por outro lado, uma correlação positiva entre a colonização por *S. pneumoniae* e *H. influenzae* já foi descrita, o que pode ter contribuído para os altos níveis de anticorpos contra *H. influenzae* encontrados na admissão (Chien et al., 2013).

As limitações deste estudo devem ser enfatizadas. Em primeiro lugar, os dados sobre o estado de colonização das crianças avaliadas não estavam disponíveis e o possível efeito da colonização por *S. pneumoniae* nos níveis de anticorpos na admissão não foi avaliado. Em segundo lugar, nosso estudo foi composto de crianças não vacinadas, o que não representa a realidade da maioria dos países na era pós-PCV. No entanto, evidências recentes sugerem que o uso de PCV não interfere no resultado de testes sorológicos utilizando em proteínas pneumocócicas entre crianças com PAC (Andrade et al., 2016), o que favorece a generalização de nossos resultados. Além disso, não tivemos dados sobre o uso de outras vacinas que poderiam ter influenciado os resultados aqui apresentados, como a vacina contra o *H. influenzae* tipo B. No entanto, a cobertura vacinal para o *H. influenzae* tipo b na população pediátrica brasileira é alta (> 80%), então diferenças nas taxas de vacinação provavelmente não afetaram nossos resultados (Rede Interagencial de Informação para a Saúde. Cobertura de Vacinas, 2008). Finalmente, como todos os antígenos de *H. influenzae* e *M. catarrhalis* foram conjugados em uma única “bead” por bactéria, não foram obtidas leituras de fluorescência individuais para esses antígenos.

Em conclusão, esta investigação demonstrou que, entre as crianças não internadas com diagnóstico clínico de PAC que foram submetidas a um raio-X de tórax, aquelas com pneumonia radiologicamente confirmada apresentaram maior freqüência de infecção por *S. pneumoniae* em comparação a crianças com raio-X de tórax normal. Além disso, a presença de infecção pneumocócica foi associada de forma independente à pneumonia

radiologicamente confirmada e o raio-X de tórax normal tem um alto valor preditivo negativo para a infecção pneumocócica.

4. Efeito da vacinação pneumocócica na colonização nasofaríngea por *S. pneumoniae, S. aureus, H. influenzae* e *M. catarrhalis*

Neste estudo, descobrimos que a vacinação com PCV10 não alterou as taxas de colonização por *S pneumoniae, H. influenzae, M. catarrhalis* e *S. aureus*. No entanto, crianças não vacinadas apresentaram maior taxa metabólica para as cepas de pneumococo colonizadoras do que nas crianças vacinadas. Além disso, uma correlação significativa entre os transcritos de mRNA e as leituras de DNA do rRNA 16S foi encontrada para *S. pneumoniae* e *H. influenzae* apenas em crianças não vacinadas. Em conjunto, esses achados indicam que a vacinação pneumocócica afeta a atividade biológica de patógenos respiratórios bacterianos em crianças.

A vacinação pneumocócica promove a diminuição da colonização por sorotipos de *S. pneumoniae* incluídos na vacina, associada simultaneamente ao aumento dos sorotipos não incluídos (Brandileone et al., 2016, Vesikari et al., 2016), o que resulta em uma freqüência geral inalterada de colonização pneumocócica (Lindstrand et al., 2016). Além disso, evidências recentes sugerem que a PCV10 não reduz as taxas de colonização por *H. influenzae* (Brandileone et al., 2016; Vesikari et al., 2016), apesar da presença da proteína D na sua composição. Também não houve diferença nas taxas de colonização por *M. catarrhalis* ou *S. aureus* após vacinação pneumocócica em um ensaio clínico avaliando a eficácia da PCV10 na colonização nasofaríngea por bactérias patogênicas (Vesikari et al., 2016). De fato, não encontramos diferença nas taxas de colonização por *S. pneumoniae, H. influenzae, M. catarrhalis* ou *S. aureus* entre crianças vacinadas ou não com PCV10. No

entanto, o uso de PCV é capaz de induzir mudanças no perfil metabólico do pneumococo durante a colonização nasofaríngea, conforme sugerido previamente por modelos matemáticos (Watkins et al., 2015). O *S. pneumoniae* possui sistemas de sinalização ligados a quinases que detectam alterações no microambiente externo e, por sua vez, permitem alterações no seu programa genético em resposta (van der Poll & Opal, 2009). Portanto, ao invés de afetar somente as taxas de colonização, a vacinação pneumocócica pode promover mudanças no comportamento biológico das bactérias colonizadoras através da mudança das características do microambiente.

Neste estudo, descobrimos que as cepas de pneumococo carreadas em crianças vacinadas com PCV10 têm uma menor taxa metabólica em comparação com aquelas em crianças não vacinadas (Tabela 3). Esta descoberta corrobora o menor potencial invasivo do pneumococo colonizador após o uso de PCV (Lindstrand et al., 2016). A redução da atividade metabólica do pneumococo em crianças vacinadas pode representar uma resposta adaptativa dessa bactéria em um ambiente hostil criado pela ativação do sistema imunológico. Além disso, as crianças não vacinadas tiveram uma correlação positiva entre a densidade de bactérias colonizadoras (leituras de DNA do rRNA 16S) e as contagens de mRNA, indicando que as bactérias carreadas estavam em um estado de replicação ativo. As crianças vacinadas, no entanto, apresentaram uma correlação fraca, o que pode representar a inibição da replicação ou mesmo a atividade bactericida do sistema imunológico.

As crianças vacinadas também apresentaram uma fraca correlação entre a carga bacteriana (leituras de DNA do rRNA 16S) e mRNA contagens de *H. influenzae*. O efeito do PCV10 na colonização nasofaríngea por *H influenzae* não foi esclarecido até o momento. Prymula et al (2009) relataram uma diminuição nas taxas de colonização por esta bactéria após a dose de reforço de uma vacina pneumocócica conjugada com Proteína D, enquanto Vesikari et al (2016) não encontraram diferença nas taxas de colonização por *H. influenzae*

entre crianças vacinadas e não vacinadas com PCV10 (Prymula et al., 2009, Vesikari et al., 2016). No Brasil, foram detectadas taxas mais elevadas de colonização por *H influenzae* em crianças vacinadas com PCV10 (Brandileone, 2016). Em nosso estudo, as taxas de colonização por esta bactéria foram extremamente altas em crianças vacinadas e não vacinadas e, portanto, não foram encontradas diferenças nas taxas e na densidade de colonização. No entanto, levantamos a hipótese de que a PCV10 produz uma resposta imunológica antígeno-específica que suprime a atividade metabólica ou leva à morte bacteriana, gerando a fraca correlação encontrada entre as leituras de DNA do rRNA 16S e transcrição de mRNA para *H. influenzae* em crianças vacinadas.

Encontramos maiores contagens de mRNA de *S. aureus* em crianças que receberam PCV10, indicando que, embora as taxas de colonização fossem semelhantes em crianças vacinadas e não vacinadas, as cepas colonizadoras em crianças vacinadas apresentavam um perfil metabólico mais ativo. Como encontramos menor atividade metabólica de *S. pneumoniae* em crianças vacinadas, este achado corrobora a associação negativa entre colonização nasofaríngea por *S. pneumoniae* e *S. aureus* (van den Bergh et al., 2012; Xu et al., 2012). Não encontramos diferença nas taxas de colonização ou na atividade metabólica de *M. catarrhalis* entre crianças vacinadas e não vacinadas com PCV10.

As limitações deste estudo devem ser observadas. O grupo de crianças vacinadas era heterogêneo, pois as crianças receberam diferentes doses de PCV10. No entanto, não encontramos nenhum efeito do número de doses de PCV10 nas taxas de detecção de transcritos de mRNA ou colonização bacteriana (leituras de DNA do rRNA 16S), conforme avaliado por regressão logística. Da mesma forma, não houve efeito do número de doses de PCV10 na densidade bacteriana ou na taxa metabólica de qualquer das bactérias estudadas. Portanto, as diferenças nos esquemas de vacinação não afetaram significativamente as variáveis de interesse desse estudo.

Em conclusão, a vacinação com PCV10 não afetou as taxas de colonização ou densidade bacteriana de colonização por *S. pneumoniae, H. influenzae, M. catarrhalis* e *S. aureus*. Contudo, encontramos menor atividade metabólica de *S. pneumoniae* em crianças vacinadas, em paralelo com maiores contagens de mRNA de *S. aureus*. Além disso, apenas as crianças não vacinadas apresentaram uma correlação positiva entre a densidade bacteriana e os transcritos de mRNA para *S. pneumoniae* e *H. influenzae*, indicando um efeito supressor ou bactericida do sistema imune em crianças vacinadas. Em conjunto, nossos dados demonstram que a vacinação com PCV10 altera a relação RNA/DNA dos patógenos no microbioma nasofaríngeo, o que pode representar uma avaliação mais sensível dos efeitos vacinais comparado com taxas quantitativas de colonização nasofaríngea.

5. Efeito da vacinação pneumocócica na detecção de resposta sorológica e desenvolvimento de complicações entre crianças com IRA

Este estudo demonstrou que a colonização nasofaríngea por *S. pneumoniae, H. influenzae e M. catarrhalis* não está associada à detecção de resposta sorológica contra as bactérias acima mencionadas em um modelo controlado por idade e uso de PCV10. Além disso, as crianças que desenvolveram OMA tiveram uma maior freqüência de resposta sorológica contra *S. pneumoniae*, apesar de não haver diferença nas taxas de colonização por esta bactéria.

Aumentos nos níveis de anticorpos contra as proteínas pneumocócicas após colonização por *S pneumoniae* foram previamente identificados em coortes prospectivas (Simell et al., 2009; Lebon et al., 2011, Hagerman et al., 2013). Este aumento na IgG anti-proteína induzida por colonização pneumocócica, no entanto, não foi suficientemente intenso para permitir a detecção de uma resposta sorológica em uma coorte de 36 crianças seguidas

durante os primeiros 2 anos de vida (Turner et al., 2013). Aqui, demonstramos a detecção de resposta sorológica contra antígenos pneumocócicos em quase 30% das crianças com IRA, a qual não foi associada à colonização atual por esta bactéria. Por outro lado, a detecção de respostas sorológicas contra antígenos protéicos de *S pneumoniae* foi descrita no contexto de infecções respiratórias bacterianas sintomáticas, como OMA (Rapola et al., 2001) e PAC (Andrade et al., 2016b). Além disso, é importante notar que a ocorrência de aumentos superiores a 2 vezes nos níveis de anticorpos anti-proteína tem boa sensibilidade e especificidade (> 90%) para pneumonia pneumocócica (Andrade et al., 2016b). Em conjunto, esses dados sugerem que a resposta imune induzida por infecções bacterianas sintomáticas é diferente da resposta imune que ocorre durante a colonização bacteriana, caso em que o aumento da IgG é breve e de menor magnitude.

Descobrimos que as crianças com OMA apresentaram maior freqüência de resposta sorológica contra *S. pneumoniae* do que crianças que não desenvolveram OMA. É importante notar que Oliveira et al (2016), em um estudo que incluiu 422 pacientes da coorte CHIADO-IVAS, relataram que o uso de PCV10 foi inversamente associado ao desenvolvimento de OMA (Oliveira et al., 2016). Como o uso de PCV reduz a incidência de OMA causada pelo pneumococo (Littorin et al., 2016), é provável que o exceedente de casos de OMA encontrados nesse estudo tenha sido causado por *S. pneumoniae*. No nosso trabalho, no entanto, o grupo de estudo avaliado foi menor devido à disponibilidade de amostras para análise sorológica. Consequentemente, não foi encontrada associação entre o uso de PCV10 e desenvolvimento de complicações. De fato, o tamanho da amostra necessário para a detecção de uma diferença estatisticamente significante da mesma magnitude que a relatada aqui (calculada como 304 pacientes, considerando que o poder do estudo foi de 80%, o erro alfa 5% e que as duas proporções testadas foram inferiores a 25% e superiores a 40%, respectivamente) é maior que a disponível para este estudo (Hulley, 1988). Portanto, nós

supomos que as crianças com OMA apresentaram maior taxa de infecção por *S pneumoniae*, sendo que a significância estatística não foi atingida devido ao tamanho da amostra.

Não foram encontradas diferenças nos níveis de IgG contra as proteínas pneumocócicas na primeira amostra de soro entre crianças que foram colonizadas ou não por *S. pneumoniae*. No entanto, é importante notar que este estudo apenas avaliou um episódio de colonização nasofaríngea e os níveis basais de anticorpos contra os antígenos pneumocócicos refletem a ocorrência de encontros prévios cumulativos com esta bactéria. Por exemplo, níveis mais baixos de IgG contra as proteínas pneumocócicas entre as crianças que receberam PCV10 refletem o menor número de episódios de colonização (Hammit et al., 2014) e doença (Tregnaghi et al., 2014) causada pela vacinação e foi relatado anteriormente em uma coorte de crianças com PAC (Andrade, 2016a). Os níveis mais elevados de IgG contra antígenos protéicos de *H. influenzae* podem ter sido causados pelo aumento da colonização por esta bactéria ao longo do tempo, que foi relatado após vacinação pneumocócica (Camili et al., 2015, Brandileone et al., 2016).

Encontramos aqui uma interação positiva entre colonização por *H. influenzae* e *M. catarrhalis*, com maior freqüência de colonização por *M. catarrhalis* em crianças já colonizadas por *H. influenzae* e vice-versa. Além disso, as crianças colonizadas por *H. influenzae* apresentaram níveis mais altos de anticorpos contra *M. catarrhalis* na primeira amostra de soro, o que sugere um efeito sinérgico de longa data para a colonização por essas bactérias. A co-colonização por *H. influenzae* e *M. catarrhalis* é um achado comum, e tem sido relatado que ocorre com mais freqüência do que a colonização por qualquer destas bactérias isoladamente (Verhaegh et al., 2011). Embora a competição bacteriana ocorra na nasofaringe (van den Bergh et al., 2012; Xu et al., 2012), foi relatado que as cepas de *M. catarrhalis* ajudam *H. influenzae* a evadir a morte celular mediada pelo complemento através da secreção de vesículas da membrana externa (Outer Membrane Vesicles - OMVs) contendo

a proteína de superfície ubíqua (Usp) A1 e UspA2, as quais interagem com o terceiro componente do sistema do complemento (C3) (Tan et al., 2007). Portanto, nossos dados reforçam a interação entre *H. influenzae* e *M. catarrhalis* na nasofaringe.

As limitações deste estudo devem ser observadas. Em primeiro lugar, a avaliação da colonização nasofaríngea foi realizada em apenas um momento, portanto nenhuma informação de colonização em uma série temporal estava disponível. No entanto, informações importantes foram extraídas dos níveis de anticorpos contra os antígenos estudados na primeira amostra de soro, os quais refletiram episódios anteriores de colonização ou infecção. Em segundo lugar, devido às limitações nos recursos, o tamanho da amostra foi inferior ao necessário para avaliar as diferenças nas taxas de resposta sorológica entre pacientes que apresentaram ou não complicações. No entanto, ainda pudemos observar uma maior taxa de resposta sorológica contra *S. pneumoniae* entre crianças que desenvolveram OMA, o que está de acordo com dados publicados anteriormente.

Em conclusão, a colonização nasofaríngea ativa o sistema imunológico, mas não é capaz de provocar respostas sorológicas semelhantes às causadas por infecções bacterianas sintomáticas. Crianças com IRA que desenvolvem OMA têm maior taxa de resposta sorológica contra *S. pneumoniae*.

6. Efeito da vacinação pneumocócica nos níveis de anticorpos basais e na frequência da resposta sorológica contra antígenos protéicos entre crianças com PAC

Nosso estudo caso-controle demonstrou que há uma diferença significativa nos níveis de anticorpos basais contra antígenos protéicos de *S. pneumoniae* e *M. catarrhalis* entre crianças com idade pareada que foram vacinadas com PCV10 ou não receberam esta vacina. No entanto, não houve diferença entre vacinados e não vacinados na freqüência de resposta sorológica contra os antígenos estudados durante um episódio de PAC.

Crianças vacinadas apresentaram valores de fluorescência significativamente menores para quatro dos antígenos pneumocócicos avaliados (Ply, PspA, StkP e PcsB). Além disso, os níveis de anticorpos contra CbpA, PcpA e PhtD foram menores em crianças vacinadas em comparação com controles não vacinados, embora significância estatística não tenha sido alcançada. Da mesma forma, no estudo de Prevaes et al, houve também uma tendência para menores leituras de fluorescência em crianças vacinadas para os antígenos Nan, Pilus A, PspA e PsaA em crianças de 24 meses de idade (Prevaes et al., 2012). Por sua vez, Ditse et al não encontraram diferença nos níveis de anticorpos contra antígenos protéicos entre crianças vacinadas com PCV7 ou não vacinadas (Ditse et al., 2013). No entanto, a população estudada por Ditse et al tinha idade mais avançada do que a avaliada neste estudo, o que pode comprometer a comparação do efeito da vacinação pneumocócica devido à diminuição da eficácia das PCVs a longo prazo após a vacinação inicial (Madhi et al., 2007). Consequentemente, nenhum efeito da vacinação sobre a prevalência da colonização total ou de sorotipos de *S. pneumoniae* incluídos na vacina foi encontrado no estudo acima mencionado (Madhi et al., 2007; Ditse et al., 2013). Além disso, nosso estudo é o primeiro a avaliar o efeito do PCV10 no nível de anticorpos basais contra antígenos protéicos em crianças vacinadas e não vacinadas. Conseqüentemente, é possível que a maior cobertura fornecida pelo PCV10 quando comparado às PCVs utilizadas em estudos anteriores possa ter influenciado a diferença nos níveis basais de anticorpos entre crianças vacinadas e não vacinadas.

Os valores mais baixos de fluorescência encontrados para crianças vacinadas podem ter sido causados pelos efeitos do PCV10 na taxa de colonização nasofaríngea. Por exemplo, o uso de PCV10 pode ter reduzido a taxa de colonização pneumocócica para as crianças estudadas, como já foi relatado em estudos anteriores com outros tipos de PCV (van Gils et al., 2009; Spijkerman et al., 2012). Nessa configuração, ao diminuir as taxas de colonização

por *S. pneumoniae*, as crianças vacinadas não foram expostas a numerosas proteínas pneumocócicas (Tai, 2006). Isso, por sua vez, poderia ter reduzido a produção de anticorpos anti-pneumocócicos nesse subgrupo de crianças. De fato, já foi demonstrado que as crianças freqüentemente colonizadas com *S. pneumoniae* apresentam níveis mais altos de anticorpos anti-proteína, em comparação com aquelas em que a colonização não foi encontrada (Pichichero et al., 2012).

Apesar das diferenças dos níveis de anticorpos, não foi encontrada diferença estatisticamente significante entre as crianças vacinadas e não vacinadas em relação à freqüência de respostas sorológicas contra as proteínas pneumocócicas estudadas. Uma possível explicação para esta descoberta é que, em crianças vacinadas, a doença tenha sido causada por sorotipos não incluídos na PCV10. Aumentos na frequência de DPI causada por sorotipos não incluídos nas vacinas foram relatados após a introdução das PCVs (Flasche et al., 2011). Os antígenos utilizados neste estudo são, no entanto, altamente conservados e quase universalmente presentes em todas as cepas de *S. pneumoniae* (Brooks-Walter et al., 1999; Adamou et al., 2001; Tai, 2006; Giefing et al., 2008; Croney et al., 2012; Khan et al., 2012a; Khan & Pichichero, 2012b). Portanto, a resposta sorológica contra eles não deve ser substancialmente afetada pelo sorotipo do pneumococo causando PAC. Além disso, altos níveis de vários anticorpos anti-proteína pneumocócica reduzem a aderência do pneumococo às células epiteliais do pulmão humano (Kaur et al., 2014) e foram associados a uma freqüência reduzida de IRA em crianças (Lebon et al., 2011; Ditse et al., 2013). Portanto, embora as crianças vacinadas com PCV10 sejam protegidas contra doenças causadas pelos sorotipos cobertos com a vacina, elas não tinham o efeito protetor de altos níveis de anticorpos anti-protéicos, tornando-as sensíveis a infecção por cepas de pneumococo dos sorotipos não incluídos nas formulações vacinais. Esse fato, por sua vez, também pode ter contribuído para a freqüência similar de resposta sorológica em crianças vacinadas e não

vacinadas. No entanto, é importante lembrar que este estudo avaliou crianças com pneumonia não-grave tratadas ambulatorialmente, população na qual os vírus são a principal causa de PAC (Bradley et al., 2011; Ruuskanen, 2011). Portanto, um menor impacto da vacinação pneumocócica é esperado neste grupo de pacientes.

Não houve diferença entre os níveis de anticorpos basais ou a freqüência de resposta sorológica contra *H. influenzae* entre crianças vacinadas e não vacinadas. Por outro lado, crianças vacinadas apresentaram níveis mais altos de anticorpos contra *M. catarrhalis* quando comparados aos não vacinados. Uma possível explicação é que as crianças vacinadas podem ter tido uma maior taxa de colonização por esta bactéria, pois a colonização pelos sorotipos *S. pneumoniae* incluídos na vacina foi prevenida e, possivelmente, permitiu uma maior taxa de colonização por *M. catarrhalis*. Mudanças na taxa de colonização nasofaríngea por *M. catarrhalis* já foram relatadas em certas circunstâncias que sabidamente afetam a flora local, como o comparecimento a creches (Verhaegh et al., 2010). No entanto, a maioria dos estudos que avaliam as taxas de colonização por *M. catarrhalis* após vacinação pneumocócica não encontrou diferença entre crianças vacinadas e não vacinadas (Van Gils et al., 2011; Dunne et al., 2012).

As limitações deste estudo devem ser reconhecidas. Em primeiro lugar, o grupo de crianças que receberam PCV10 foi pequeno e heterogêneo quanto à conclusão do esquema vacinal e ao número de doses de PCV10 administradas. No entanto, não encontramos nenhum efeito do número de doses de PCV10 ou do intervalo de tempo entre a primeira/última dose de vacina e a primeira coleta de amostras de soro sobre os níveis de anticorpos basais ou na freqüência de resposta sorológica neste grupo de crianças. Por sua vez, isso poderia sugerir que embora o grupo de crianças vacinadas fosse efetivamente heterogêneo, as diferenças dos esquemas de vacinação não afetaram significativamente as variáveis de interesse deste estudo. Além disso, os grupos de crianças vacinadas e seus

respectivos controles não vacinados foram muito semelhantes em relação à idade e outros aspectos clínicos, de modo que as diferenças aqui encontradas não podem ser explicadas por esses fatores. É importante lembrar que este foi um estudo exploratório, e nossos achados devem ser confirmados em estudos com uma amostra maior e homogênea em relação ao estado vacinal. Em segundo lugar, não tivemos dados do estado de colonização das crianças avaliadas ou testes definitivos sobre a etiologia da PAC. Portanto, a associação entre os achados deste estudo e as possíveis mudanças nas taxas de colonização ou etiologia da PAC são apenas teóricas. Finalmente, não tivemos dados sobre o uso de outras vacinas que possam ter afetado as taxas de colonização ou infecção pelos agentes patogênicos estudados, como a vacina contra o *H. influenzae* tipo b (Hib). No entanto, já foi demonstrado que a cobertura da vacina Hib é alta na população pediátrica no Brasil, portanto provavelmente não afetou os resultados deste estudo (Rede Interagencial de Informação para a Saúde. Cobertura de Vacinas, 2008).

Em conclusão, demonstramos que as crianças que foram vacinadas com PCV10 apresentaram níveis mais baixos de anticorpos basais contra quatro antígenos protéicos de *S. pneumoniae* quando comparados a crianças não vacinadas com idade e apresentação clínica semelhantes. Isso poderia refletir menores taxas de colonização de *S. pneumoniae* em crianças vacinadas como conseqüência da vacinação com PCV10. Apesar das diferenças nos níveis de anticorpos basais, não houve diferença nas taxas de resposta sorológica contra antígenos protéicos de *S. pneumoniae, H. influenzae* e *M. catarrhalis* entre crianças vacinadas e não vacinadas com PAC.

7. Considerações finais

Neste trabalho, fizemos uma análise abrangente de métodos diagnósticos para infecção respiratória em crianças com ênfase no *Streptococcus pneumoniae*, um agente patogênico responsável por até 11% dos óbitos em crianças com idade inferior a 5 anos

(O'Brien, 2009). Foi realizada a validação de um novo teste sorológico trazendo duas inovações tecnológicas para o diagnóstico de infecção por *S. pneumoniae:* a inclusão de novos antígenos protéicos, que permitem a detecção de respostas sorológicas ao pneumococo independentemente do sorotipo infectante (Tai, 2006); e o teste simultâneo de diversos antígenos (teste em multiplex). Esta estratégia gerou um incremento na sensibilidade do teste desenvolvido, como de fato observamos uma sensibilidade >90% sem gerar um decréscimo correspondente na especificidade (a qual também foi >90%) (Artigo 1). Uma adaptação do teste sorológico desenvolvido para avaliar avidez também se mostrou uma técnica promissora, capaz de diferenciar casos com e sem doença pneumocócica (Artigo 2). Verificamos também que a detecção de respostas sorológicas não sofre influência de uma colonização momentânea, eliminando assim um possível viés para a interpretação dos resultados de testes sorológicos para doença pneumocócica (Artigo 5). Além disso, a forte associação encontrada entre resposta sorológica ao pneumococo utilizando o teste aqui desenvolvido e a presença de pneumonia radiologicamente confirmada reforça a validade do nosso teste, além de trazer importantes implicações para o manejo clínico de crianças com PAC (Artigo 3). Finalmente, ao avaliar o efeito da vacinação sobre nosso teste sorológico, constatamos que não há efeito da vacinação pneumocócica sobre a frequência de detecção de resposta sorológica ao pneumococo (Artigo 6).

Curiosamente, encontramos níveis basais de anticorpos contra antígenos protéicos de *S. pneumoniae* em crianças vacinadas com PCV10 mais baixos do que em crianças não vacinadas (Artigo 6). Este achado foi consistente tanto no grupo de crianças com PAC (Artigo 6) quanto no grupo de crianças com IRA (Artigo 5). Hipotetizamos que esta diferença seja devido a maior quantidade de episódios de colonização ou doença devido ao pneumococo em crianças não vacinadas. No entanto, não encontramos diferenças nas taxas brutas de colonização pelo pneumococo em crianças vacinadas ou não vacinadas com

PCV10. Pelo contrário, foi inovador notar que a vacinação pneumocócica pode atuar através da modificação do comportamento biológico de *S. pneumoniae*, suprimindo sua atividade metabólica (Artigo 4). Desse modo, infere-se que outras variáveis devem ser levadas em consideração ao se avaliar os efeitos imunológicos da vacinação pneumocócica, como por exemplo, alterações na afinidade do anticorpo e efetividade da ação bactericida (que poderiam ser avaliadas através de testes de avidez ou opsonofagocitose).

Assim, concluímos que testes sorológicos são ferramentas valiosas para diagnóstico de infecção pneumocócica e compreensão da resposta imune ao *S. pneumoniae* e à vacinação para este patógeno, cujos efeitos no ciclo colonização-infecção ainda devem ser completamente elucidados.

XI. CONCLUSÃO

1) O teste sorológico desenvolvido com múltiplas proteínas pneumocócicas é um método promissor para o diagnóstico de infecção pneumocócica em crianças com pneumonia, com especificidade e sensibilidade superiores a 90%. Um ponto de corte de aumento ≥1,52 vezes no nível de anticorpos basais deve ser considerado para PcpA, enquanto que um ponto de corte ≥2 vezes deve ser usado para Ply, CbpA, PspA1 e 2, PhtD, StkP-C e PcsB- N.

2) Desenvolvemos um ensaio de avidez robusto em multiplex usando proteínas pneumocócicas. O teste foi capaz de identificar diferenças na avidez de IgG anti-proteína entre grupos de crianças com e sem doença pneumocócica, com menor avidez encontrada no grupo de crianças com doença pneumocócica. StkP-C e PcpA devem ser considerados para inclusão em testes de avidez projetados para o diagnóstico de doença pneumocócica.

3) Crianças com pneumonia radiologicamente confirmada têm maior freqüência de infecção por *S. pneumoniae* em comparação com crianças com raio X de tórax normal. A presença de infecção pneumocócica é associada de forma independente à pneumonia radiologicamente confirmada e o raio-X de tórax normal possui alto valor preditivo negativo para a infecção pneumocócica.

4) A vacinação com PCV10 não afetou as taxas ou a densidade de colonização por *S. pneumoniae, H. influenzae, M. catarrhalis* e *S. aureus*. Foi encontrada atividade metabólica mais baixa para *S. pneumoniae* em crianças vacinadas, em paralelo com maiores contagens de RNA para *S. aureus*. Apenas crianças não vacinadas apresentaram uma correlação positiva entre a carga bacteriana e os transcritos de mRNA para *S. pneumoniae* e *H. influenzae*, indicando um efeito supressor ou bactericida do sistema imune em crianças vacinadas.

5) A colonização nasofaríngea ativa o sistema imunológico, mas não é capaz de provocar respostas sorológicas semelhantes às causadas por infecções bacterianas sintomáticas. Crianças com IRA que desenvolvem OMA têm maior taxa de resposta sorológica contra *S. pneumoniae*.

6) As crianças que foram vacinadas com PCV10 apresentaram níveis mais baixos de anticorpos basais contra antígenos protéicos de *S. pneumoniae* quando comparados a crianças não vacinadas com idade e apresentação clínica semelhantes. Nenhuma diferença foi encontrada nas taxas de resposta sorológica contra antígenos protéicos de *S. pneumoniae*, *H. influenzae* e *M. catarrhalis* entre crianças vacinadas e não vacinadas com PAC.

XIII. PERSPECTIVAS DE ESTUDO

Após a realização do trabalho apresentado nesta tese, algumas perspectivas de estudo são interessantes para continuação da linha de pesquisa desenvolvida:

1) Avaliação do efeito da colonização nasofaríngea por *S. pneumoniae, H. influenzae, M. catarrhalis e S. aureus* no desenvolvimento de resposta sorológica e alterações de avidez

Este trabalho consistiria numa coorte de crianças com idade entre 6 meses e 5 anos (período de maior colonização bacteriana nasofaríngea e após o período de maior efeito de anticorpos maternos) acompanhadas no serviço de pediatria do Complexo Pediátrico Professor Hosanah de Oliveira (CPPHO/C-HUPES) para a realização de cirurgias eletivas. Amostras seriadas de aspirado nasofaríngeo e sangue seriam coletadas em um intervalo de 2 a 4 semanas por um período de 6 meses. As amostras coletadas seriam então submetidas à avaliação sorológica e ao teste de avidez com antígenos protéicos descritos nesta tese. A coleta seriada de amostras permitiria o diagnóstico da aquisição de uma nova cepa colonizadora, que por sua vez teria maior impacto sobre o sistema imune e pode representar um viés na interpretação de testes sorológicos.

2) Avaliação da interação entre patógenos bacterianos e virais na nasofaringe de crianças com infecção respiratória aguda

Como comentado previamente nesta tese, diversos patógenos coexistem dinamicamente na nasofaringe de crianças. Com o advento de uma infecção respiratória aguda, esta rede de interrelações se altera, de modo que infecções por determinados agentes virais podem propiciar a colonização por diferentes bactérias e vice-versa. Para este trabalho, as amostras

de aspirado nasofaríngeo de crianças com infecção respiratória aguda seriam submetidas a testes moleculares (Nanostring) para a detecção de mRNA de agentes virais. A avaliação da relação entre diferentes agentes virais e bacterianos seria realizada atreves da correlação entre dados quantitativos e qualitativos da detecção de cada agente na amostra de aspirado nasofaríngeo.

3) Avaliação do efeito da colonização bacteriana sobre características clínicas em crianças com infecção respiratória aguda

A colonização nasofaríngea por bactérias é considerado um passo fundamental para o desenvolvimento de infecções respiratórias invasivas. No entanto, também se hipotetiza que o estado de colonização nasofaríngea pode alterar características clínicas durante o curso de uma infecção viral. Bactérias colonizadoras teriam este efeito por meio do estímulo inflamatório local e sistêmico gerado pela colonização. Para este estudo, amostras de soro seriam analisadas para a detecção de citocinas de perfil TH1, TH2 e TH17, e dados clínicos seriam associados à presença de colonização pelas bactérias avaliadas e pelas alterações imunológicas promovidas.

4) Avaliação do papel de agentes virais como causa de pneumonia adquirida na comunidade em crianças menores de 5 anos

Após a avaliação do papel de agentes bacterianos típicos como agentes de PAC em crianças menores de 5 anos, procederíamos à análise de agentes virais (VSR, adenovírus, influenza, parainfluenza e rinovírus) através da pesquisa de antígeno em secreção nasal e também da

pesquisa de anticorpos específicos em soros pareados. É esperado que agentes virais sejam responsáveis pela maior parte dos casos de PAC nesta coorte.

XIII. SUMMARY

Community acquired pneumonia (CAP) is a common acute respiratory infection (ARI) in childhood, and has high morbidity and mortality rates. We performed a comprehensive analysis of factors associated with diagnosis of infection by *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis* among children with CAP. Firstly, we validated a multiplex serological assay in multiplex with eight protein antigens from *S. pneumoniae* (Ply, CbpA, PspA 1 and 2, PcpA, PhtD, StkP and PcsB) using positive and negative controls. We found the the described assay was highly sensitive and specific for invasive pneumococcal infection, and that an increase ≥ 2fold in the basal antibody levels is an adequate cut-off for diagnosis for 7 of the tested proteins. Secondly, we developed an avidity assay using pneumococcal proteins, which was able to reliably distinguish samples from children with and without pneumococcal disease. We also assessed the role of the chest radiograph as a diagnostic tool for children with CAP who had serum saples tested for infection by either *S. pneumoniae*, *H. influenzae* and *M. catarrhalis.* Children with radiologically confirmed pneumonia presented a higher rate of infection by *S. pneumoniae* compared to children with a normal chest radiograph. Furthermore, the presence of a normal chest radiograph had high negative predictive value for infection by *S. pneumoniae.* The effect of pneumococcal vaccination on serological data was also evaluated. We found no difference in the rates of antibody response detection against *S. pneumoniae, H. influenzae* or *M. catarrhalis* between children who received 10-valent pneumococcal conjugate vaccine (PCV10) or not. However, vaccinated children presented lower levels of IgG against protein antigens from *S. pneumoniae* compared to unvaccinated children.

Subsequently, we assessed the role of *S. pneumoniae, H. influenzae* and *M. catarrhalis* in nasopharyngeal colonization, a fundamental step in the development of invasive bacterial infection. There was no association between nasopharyngeal colonization and detection of

antibody responses against the tested bactéria. When the effect of use of PCV10 on nasopharyngeal flora was evaluated, we found that vaccinated children had lower metabolic rate for *S. pneumoniae* than unvaccinated children, though no difference in carriage rates was found for the studied bactéria.

XIV. REFERÊNCIAS

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