Universidade do Estado do Rio de Janeiro
Centro Biomédico
Faculdade de Ciências Médicas

Mônica de Cássia Firmida

Impacto clínico da infecção por *Achromobacter xylosoxidans* na fibrose cística

Rio de Janeiro
2016

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Tese apresentada, como requisito parcial para obtenção do título de Doutor, ao Programa de Pós-Graduação em Ciências Médicas, da Universidade do Estado do Rio de Janeiro.

Orientadores: Prof. Dr. Agnaldo José Lopes

Prof.ª Dra. Elizabeth de Andrade Marques

Rio de Janeiro

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Aprovada em 23 de novembro de 2016.

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Rio de Janeiro
2016

DEDICATÓRIA

Aos portadores de fibrose cística e seus familiares.

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Há muros que só a paciência derruba.
E há pontes que só o carinho constrói.

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RESUMO

FIRMIDA, Mônica de Cássia. *Impacto clínico da infecção porAchromobacter xylosoxidans na fibrose cística.* 2016. 93 f. Tese (Doutorado em Ciências Médicas) – Faculdade de Ciências Médicas, Universidade do Estado do Rio de Janeiro, Rio de Janeiro, 2016.

A frequência de infecção por *Achromobacter xylosoxidans* na fibrose cística, tem aumentado nos últimos anos. Porém, o impacto clínico da infecção por este microrganismo ainda é controverso. Este estudo retrospectivo teve como objetivo avaliar o impacto clínico da infecção por *A. xylosoxidans* em três grupos de pacientes com fibrose cística: indivíduos infectados cronicamente (n = 10), intermitentemente (n = 15) e nunca infectados por *A. xylosoxidans* (n = 18), todos infectados cronicamente por *Pseudomas aeruginosa*. Os três grupos foram transversalmente avaliados em dois momentos: no momento da infecção pelo *A. xylosoxidans* (M1) e 2 anos depois (M2). O grupo nunca infectado para *A. xylosoxidans* foi pareado com os outros dois grupos por idade (±1 ano) e sexo. Características demográficas, dados clínicos, de função pulmonar, e coinfecções bacterianas crônicas foram avaliados. Da população total do estudo, 87% tinham menos de 18 anos e 65,1% eram do sexo feminino. Os indivíduos cronicamente infectados por *A. xylosoxidans* tiveram menor valor de volume expiratório forçado no primeiro segundo desde o ínicio do estudo (51,7% no grupo com infecção crônica, 82,7% no grupo com infecção intermitente e 76% no grupo não infectado) e maior frequência de coinfecção por *Staphylococcus aureus* resistente à meticilina (P = 0,002). Em 2 anos, a queda da função pulmonar não foi significativa em nenhum dos três grupos, embora tenha sido observada tendência à diminuição significativa no grupo cronicamente infectado por *A. xylsosoxidans*. A média anual de hospitalizações também foi maior neste grupo (P = 0,033). Em suma, neste estudo a infecção por *A. xylosoxidans* foi mais frequente nos pacientes pediátricos (<18 anos) e a função pulmonar do grupo cronicamente infectado por este microrganismo foi pior do que a dos outros grupos desde o início do estudo. No entanto, não detectamos impacto clínico, com exceção da maior frequência de hospitalizações em pacientes cronicamente colonizados por *A. xylosoxidans.*

Palavras-chave: Achromobacter. Fibrose cística.

ABSTRACT

FIRMIDA, Mônica de Cássia. *Clinical impact of Achromobacter xylosoxidans infection in cystic fibrosis.* 2016. 93 f. Tese (Doutorado em Ciências Médicas) – Faculdade de Ciências Médicas, Universidade do Estado do Rio de Janeiro, Rio de Janeiro, 2016.

The frequency of *Achromobacter xylosoxidans* infection in cystic fibrosis has increased in recent years. However, the clinical impact of the infection with this microorganism is still controversial. This retrospective study aimed to evaluate the clinical impact of *A. xylosoxidans* infection in three groups of patients with cystic fibrosis: chronically (n = 10), intermittently (n = 15) and never infected individuals (n = 18), all also chronically infected with *Pseudomonas aeruginosa.* The three groups were transversely evaluated in two moments: at the time of *A. xylosoxidans* infection (M1) and two years later (M2). The group never infected with *A. xylosoxidans* was paired with the other two groups by age (± 1 year) and sex. Demographic data, clinical data, lung function, and chronic bacterial coinfections were evaluated. Of the total study population, 87% were under 18 years and 65.1% were female. Individuals chronically infected with *A. xylosoxidans* had lower volume forced expiratory in one second since the beginning of the study (51.7% in the group with chronic infection, 82.7% in the group with intermittent infection and 76% in the group not infected) and higher frequency of coinfection with methicillin-resistant *Staphylococcus aureus* (P = 0.002). In two years, the decline in lung function was not significant in any of the three groups, although it has been observed tendency to significant decrease in the *A. xylsosoxidans* chronically infected group. The average of annual hospitalizations was also higher in this group (P = 0.033). In summary, *A. xylosoxidans* infection was more common in pediatric patients (<18 years) and pulmonary function values in the *A. xylosoxidans* chronically infected group were worse since the beginning of this study. However, we did not detect clinical impact, except for higher frequency of hospitalizations in patients chronically colonized with *A. xylosoxidans*.

Keywords: Achromobacter. Cystic fibrosis.

LISTA DE ABREVIATURAS E SIGLAS

BGNNF	Bacilo Gram-negativo não fermentador
CBc	Complexo *Burkholderia cepacia*
CFTR	*Cystic fibrosis transmembrane condutance regulator*
CVF	Capacidade vital forçada
DNA	*Deoxyribonucleic acid*
DPN	Diferença de potencial nasal
DRFC	Diabetes relacionado à fibrose cística
ENaC	Canal de sódio epitelial
EUA	Estados Unidos da América
FC	Fibrose cística
Fiocruz	Fundação Oswaldo Cruz
HUPE	Hospital Universitário Pedro Ernesto
IFF	Instituto Nacional de Saúde da Mulher, da Criança e do Adolescente Fernandes Figueira
IL1-β	Interleucina 1-beta
IL-6	Interleucina 6
IL-8	Interleucina 8
IMC	Índice de massa corporal
LBACT	Laboratório de Bacteriologia
MLST	*Multilocus sequence typing*
MRSA	*Staphylococcus aureus* resistente à meticilina
M1	Momento 1
M2	Momento 2
PCR	*Polymerase chain reaction*
PFGE	*Pulsed-field gel eletroforesis*
PPC	Policlínica Piquet Carneiro
REBRAFC	Registro Brasileiro de Fibrose Cística
SCV	*Small colony variant*
SD	*Standard deviation* (desvio padrão)
TCLE	Termo de consentimento livre e esclarecido
TIR	Tripsinogênio imunorreativo

TNF-α	Fator de necrose tumoral alfa
UERJ	Universidade do Estado do Rio de Janeiro
VEF_1	Volume expiratório forçado no primeiro segundo

LISTA DE SÍMBOLOS

%	Porcentagem
mEq/L	Miliequivalentes por litro
Kg	Quilograma

SUMÁRIO

INTRODUÇÃO

Fibrose cística (FC) é a doença genética, sistêmica e progressiva, que causa elevada morbidade para os indivíduos e que, às vezes leva a morte precoce, principalmente por evolução da doença pulmonar (CYSTIC FIBROSIS FOUNDATION, 2015; GBEFC, 2015; ZOLIN; MCKONE; VAN RENS J, 2016). O comprometimento pulmonar caracteriza-se por obstrução das vias aéreas, infecções crônicas e intensa resposta inflamatória. As infecções começam cedo e são as principais determinantes da evolução da doença pulmonar. Lactentes e pré-escolares costumam ser colonizados por *Haemophilus influenzae*, *Staphylococcus aureus* e, eventualmente, por *Pseudomonas aeruginosa*, bactéria mais prevalente na FC. *P. aeruginosa* e bactérias do complexo *Burkholderia cepacia* (CBc) são mais frequentes em adolescentes e adultos e estão claramente associadas a agravamento da doença (GBEFC, 2015; SALSGIVER et al., 2016). No entanto, a epidemiologia microbiológica na FC tem se tornado cada vez mais complexa, caracterizada pelo surgimento de novos patógenos e por maior frequência de microorganismos multirresistentes (LIPUMA, 2010; SALSGIVER et al., 2016). *Staphylococcus aureus* resistentes a meticilina (MRSA), *Burkholderia gladioli* e outras espécies do CBc, *Stenotrophomonas maltophilia*, *Achromobacter xylosoxidans*, *Ralstonia* spp. *e Pandorea* spp. são exemplos de germes emergentes (MAHENTHIRALINGAM, 2014; PARKINS; FLOTO, 2015). A evolução do conhecimento em relação aos germes emergentes pode beneficiar os indivíduos com FC. Estudo recente evidenciou alta frequência de infecção por *A. xylosoxidans* em pacientes portadores de FC acompanhados no Rio de Janeiro, porém esta população ainda não foi estudada quanto ao possível impacto clínico desta infecção (PEREIRA et al., 2011). Estudar estes aspectos relacionados à infecção por *A. xylosoxidans* nestes indivíduos é fundamental para que, se necessárias, medidas de prevenção e tratamento possam ser tomadas oportunamente, visando preservar a sua saúde e promover qualidade de vida.

1 **REVISÃO DA LITERATURA**

1.1 **Fibrose cística: aspectos gerais**

FC é a doença genética de herança autossômica recessiva e potencialmente limitadora da vida mais comum no mundo (ELBORN, 2016). Mutações localizadas no braço longo do cromossoma 7 comprometem a *cystic fibrosis transmenbrane condutance regulator* (CFTR), uma proteína grande e complexa localizada na membrana apical das células que funciona principalmente como um canal de cloreto, mas que também modula outros canais iônicos e secreta bicarbonato (DE BOECK; AMARAL, 2016; LOPES-PACHECO, 2016; MALL; HARTL, 2014)

O espectro clínico é amplo e, em muitos aspectos, é resultado do grau de déficit de CFTR (STERN, 1997; WALLIS, 1997). Os sítios mais sensíveis à sua disfunção são o sistema reprodutor masculino, as glândulas sudoríparas, o sistema respiratório e o pâncreas exócrino (ELBORN, 2016). A maioria dos homens com FC têm azoospermia obstrutiva associada a anomalia do desenvolvimento dos ductos deferentes. A esterilidade pode ser a única manifestação clínica em formas atípicas da doença (GROMAN et al., 2005; KANAVAKIS et al., 1998). Nas glândulas sudoríparas, a disfunção da CFTR causa perda excessiva de eletrólitos, em especial de cloreto. O suor salgado às vezes é uma queixa clínica e faz a FC também ser popularmente conhecida como “doença do beijo salgado”. Desidratação e distúrbios eletrolíticos ocasionalmente ocorrem em consequência deste mesmo problema. A medida da concentração de cloretos no suor, em amostra obtida por estímulo com iontoforese com pilocarpina, caracteriza o teste do suor, que é o exame mais sensível e mais acessível para a confirmação diagnóstica de FC (FARRELL et al., 2008; ROSENSTEIN; CUTTING, 1998). A insuficiência pancreática exócrina resulta de disfunções mais graves da CFTR e manifesta-se mais comumente como síndrome disabsortiva e comprometimento nutricional secundário, ocorrendo em cerca de 85% dos portadores de FC (WALKOWIAK; LISOWSKA; BLASZCZYŃSKI, 2008). A expressão clínica respiratória costuma ser mais intensa quando há déficits maiores de CFTR, mas esta relação não é linear e sofre a influência de diversos outros fatores (ELBORN, 2016).

Atualmente, há mais de 2000 mutações identificadas como possíveis causadoras de FC (CYSTIC FIBROSIS MUTATION DATABASE, 2016). Apesar desta ampla diversidade, nem todas têm significado clínico definido e menos de 25% têm incidência maior que 0,1% (CASTELLANI et al., 2008; SOSNAY; RARAIGH; GIBSON, 2016). A mutação mais frequente é a deleção de três pares de bases da fenilalanina na posição 508 da proteína (F508del), que leva à síntese de proteína CFTR anômala que, consequentemente, é quase toda destruída no complexo de Golgi, antes de chegar na membrana celular (DE BOECK; AMARAL, 2016; LOPES-PACHECO, 2016; SOSNAY; RARAIGH; GIBSON, 2016).

De acordo com os mecanismos moleculares com que levam ao déficit de CFTR, as mutações de FC são divididas em sete classes:

a) Classe I: defeito na produção da proteína. Mutações desta classe (*nonsense mutations*) podem criar um códon de terminação prematuro, em que a transcrição do RNAm é interrompida, levando à síntese de uma proteína truncada (cortada prematuramente), que é degradada rapidamente no citoplasma.
b) Classe II: defeito no processamento e no tráfico para a superfície celular, com destruição da proteína no complexo de Golgi. A mutação mais frequente na FC, F508del, é a principal desta classe.
c) Classe III: defeito na abertura do canal iônico. A mutação G551D é a mais prevalente deste grupo.
d) Classe IV: defeito na condutância do cloreto. A maioria das mutações desta classe faz com que haja função residual da CFTR.
e) Classe V: o defeito leva à redução da quantidade da proteína CFTR funcionante.
f) Classe VI: o defeito leva à síntese de CFTR de baixa estabilidade.
g) Classe VII: descrita mais recentemente, corresponde a grandes deleções genéticas com formação de proteína CFTR anômala, consequentemente destruída (DE BOECK; AMARAL, 2016).

Algumas vezes as mutações influenciam em mecanismos de mais de uma classe, como ocorre com a própria F508del: uma mutação de classe II que também compromete a abertura do canal iônico, como ocorre nas mutações de classe III (DE BOECK, AMARAL, 2016). Esta classificação ajuda no entendimento das manifestações clínicas (fenótipo) da doença, mas tem como grande objetivo auxiliar no desenvolvimento de novas drogas para tratamentos personalizados, especificamente direcionados ao defeito molecular, como corretores e

potencializadores da CFTR. Estes tratamentos são promissores para evitar a progressão da doença e promover maior qualidade de vida aos portadores de FC (DE BOECK, AMARAL, 2016; LOPES-PACHECO, 2016).

Mutações de classe I, II, III e VII geralmente estão associadas a formas mais graves da FC, em que as manifestações clínicas são mais ricas e a insuficiência pancreática está presente. Nessas, a precocidade e a exuberância das manifestações, tais quais sintomas da doença sinopulmonar, esteatorreia, comprometimento nutricional e perda excessiva de cloretos no suor, favorecem que o diagnóstico clínico ocorra mais cedo. Já mutações de classe IV a VI resultam em menor déficit da CFTR e levam a manifestações clínicas mais leves e, às vezes, até de um único órgão (por exemplo, pancreatite recorrente e infertilidade masculina por azoospermia obstrutiva) (SOSNAY, 2016). Indivíduos com quadros mais leves de FC frequentemente não têm insuficiência pancreática, apresentam melhor estado nutricional, progressão mais lenta da doença e costumam ser diagnosticados mais tarde (SIMMONDS, 2013). As mutações, no entanto, não são o único determinante da expressão clínica e da gravidade da FC. Até irmãos, com o mesmo genótipo, têm expressão clínica e evolução distintas (CASTELLANI et al., 2008). Diferentes fatores podem ser responsáveis por esta variabilidade, incluindo genes modificadores, fatores ambientais (como por exemplo, colonizações respiratórias, gênero, exposição a tabaco ou a outros poluentes ambientais, fatores climáticos), socioeconômicos, culturais, familiares, acesso a serviços de saúde e adesão ao tratamento (COLLACO, CUTTING, 2008; SCHECHTER, 2003; 2011).

A suspeita de FC se dá diante de qualquer manifestação clínica compatível, rastreamento neonatal positivo ou história familiar de FC em irmão. Existem diferentes protocolos de rastreamento neonatal no mundo. No Brasil, o mais utilizado se baseia na dosagem do tripsinogênio imunorreativo (TIR) no sangue colhido por papel filtro no “teste do pezinho” (LEÃO; AGUIAR, 2008; WALLIS, 1997). Se na primeira dosagem o nível de TIR estiver acima dos valores de referência, a avaliação deve ser prontamente repetida, antes que a criança complete 30 dias de vida. Quando o resultado permanece elevado na segunda dosagem caracteriza-se a suspeita de FC. Este rastreamento (TIR/TIR) é muito importante para a precocidade do diagnóstico. Porém, este exame tem frequência elevada tanto de resultados falso-positivos como de falso-negativos (LEÃO; AGUIAR, 2008). A confirmação diagnóstica requer evidências da disfunção CFTR: elevação da concentração de cloretos no suor (≥60 mEq/l, em duas dosagens em dias diferentes), identificação de duas mutações

genéticas causadoras da doença ou exames que mostram alteração iônica do epitélio, como a medida da diferença de potencial nasal (DPN). Concentração de cloretos no suor entre 40 e 59 mEq/L, ou entre 30 e 59 mEq/l em crianças menores de 6 meses, é considerada limítrofe, podendo ser causada por formas atípicas de FC ou por outro problema (FARRELL et al., 2008; ROSENSTEIN; CUTTING, 1998).

1.2 **Epidemiologia**

A frequência da FC e a distribuição das mutações variam de acordo com a origem étnica da população, predominando em caucasianos descendentes de europeus (CASTELLANI et al., 2008). Acredita-se que haja entre 70.000 e 100.000 indivíduos acometidos por FC no mundo (CYSTIC FIBROSIS WORLDWIDE, 2016), sendo aproximadamente 30.000 nos Estados Unidos da América (EUA) (CYSTIC FIBROSIS FOUNDATION, 2015) e 40.000 na Europa (ZOLIN; MCKONE; VAN RENS J, 2016). Nestas regiões, a incidência é estimada em 1:2.000 a 1:3.500 nascidos vivos. No Brasil, esta taxa foi estimada em 1:7.576 nascidos vivos em estudo feito envolvendo cinco estados, podendo esta frequência variar muito entre diferentes regiões (RASKIN et al., 2008). Dados referentes ao ano de 2014 mostram 3.511 pacientes com FC registrados no Brasil (GBEFC, 2015), o que certamente é um dado subestimado. Além do subregistro e do subdiagnóstico, a extensão territorial do país e a diversidade de origem étnica da população brasileira desafiam os estudos em genética e epidemiologia da FC no Brasil (MOTA et al., 2016).

A evolução do conhecimento e progressos em relação ao diagnóstico e tratamento têm levado a um aumento progressivo na sobrevida de indivíduos com FC, embora esta doença ainda seja importante causa de morte precoce (ELBORN, 2016). A mediana de sobrevida atual dos afetados é de 39,3 anos nos EUA (CYSTIC FIBROSIS FOUNDATION, 2015). Para pacientes nascidos a partir do ano 2000, esta estimativa é de ultrapassar os 50 anos (DODGE et al., 2007). A população adulta com FC tem crescido progressivamente e, em alguns países, já é maior do que a pediátrica (BURGEL et al., 2015; SIMMONDS, 2013). Apesar de acometer vários órgãos, a grande maioria dos óbitos por FC decorre do acometimento pulmonar progressivo (CYSTIC FIBROSIS FOUNDATION, 2015; GBEFC, 2015; ZOLIN; MCKONE; VAN RENS J, 2016). Com a maior sobrevida dos portadores,

outros fatores têm influenciado o prognóstico, incluindo: complicações da FC cuja incidência aumenta no decorrer da vidadiabetes relacionado a FC - DRFC, hepatopatia, osteoporose/osteopenia, hemoptise ou pneumotórax, depressão), complicações do tratamento (por exemplo, doença renal e hipoacusia secundárias a uso de aminoglicosídios), doenças relacionadas ao próprio envelhecimento populacional (por exemplo, problemas cardiovasculares), entre outros (SIMMONDS, 2013).

1.3 **A doença pulmonar**

No trato respiratório, a disfunção da CFTR leva à redução da secreção de cloretos e bicarbonato no líquido de superfície das vias aéreas. Em paralelo, a absorção do sódio aumenta através de um canal de sódio epitelial que não sofre regulação pela CFTR (ENaC). Isto provoca desidratação do líquido de superfície, o que torna o muco mais espesso e dificulta o *clearance* mucociliar, levando à obstrução das vias aéreas. A acidez contribui para o aumento da viscosidade do muco e compromete as defesas antibacterianas. Este parece ser o início do ciclo vicioso de obstrução, infecções e inflamação crônicas das vias aéreas, que são o cerne da fisiopatologia da doença pulmonar na FC (MALL; HARTL, 2014).

Citocinas próinflamatórias aumentadas, como interleucinas (IL1-β IL-6, IL-8) e fator de necrose tumoral (TNF-α), assim como influxo de neutrófilos, são características marcantes desta inflamação. Os neutrófilos agem contra as bactérias liberando e ativando enzimas, como metaloproteases e elastase neutrofílica (WAGNER; SCHULTZ; MALL, 2016). Fisiologicamente, a ação da elastase neutrofílica é modulada pela alfa-1-antitripsina que se liga à elastase impedindo-a de destruir a elastina. Na FC, a atividade da elastase neutrofílica livre acima de um limiar crítico está subjacente ao mecanismo de desenvolvimento de bronquiectasias (SLY et al., 2013). Apesar de muitas especulações, os estudos ainda são controversos ao tentar esclarecer se este estado hiperinflamatório surge após as infecções ou se as precede (LIVRAGHI-BUTRICO et al., 2012).

As infecções começam cedo. Embora os indivíduos estejam expostos a uma ampla variedade de microrganismos, um grupo relativamente limitado de bactérias são esperados na FC, destacando-se *Staphylococcus aureus*, *Haemophilus influenzae*, *Pseudomonas*

aeruginosa e bactérias do complexo *Burkholderia cepacia* (CYSTIC FIBROSIS FOUNDATION, 2015; GBEFC, 2015; HAUSER et al., 2011; ZOLIN; MCKONE; VAN RENS J, 2016). A microbiologia pulmonar na FC é muito dinâmica, caracterizada por mudanças constantes. No entanto, segue certa cronologia no decorrer dos anos de vida, apesar das particularidades individuais e de cada centro de referência. Nas crianças, predominam as infecções por *S. aureus* e *H. influenzae*. *P. aeruginosa* costuma surgir um pouco mais tarde e, atualmente, acomete pouco mais de 20% das crianças menores de 6 anos e de 70% dos adultos entre 25 e 44 anos (GBEFC, 2015; SALSGIVER et al., 2016). A frequência de *P. aeruginosa* diminuiu em relação a anos anteriores, provavelmente devido à prática de tratamento visando a erradicação e à maior diversidade de antibióticos disponíveis para tratamento da infecção crônica (SALSGIVER et al., 2016). Porém, esta ainda é a bactéria mais prevalente na FC e com grande importância na morbimortalidade associada à doença pulmonar. A infecção inicial costuma ser por cepas não mucoides, suscetíveis a maioria dos antibióticos usados em seu tratamento. Durante a infecção crônica, sob pressão de antibióticos e estresse oxidativo, a *P. aeruginosa* costuma sofrer adaptações transformando-se no fenótipo mucoide que é mais resistente aos antibióticos e à resposta imune do hospedeiro (LORÈ et al., 2012). O CBc tem 20 espécies incluídas até o momento. Algumas, especialmente a *B. cenocepacia*, têm alto potencial de patogenicidade e transmissibilidade, e estão associadas com alta morbimortalidade, enquanto outras precisam ser melhor estudadas (PARKINS; FLOTO, 2015; ZLOSNIK et al., 2015).

Nas últimas décadas, têm aumentado tanto a diversidade como a frequência de microrganismos multirresistentes identificados na FC (LIPUMA, 2010; SALSGIVER et al., 2016). Estes microrganismos emergentes têm em comum a necessidade de esclarecimento quanto ao seu papel na patogenia e no curso clínico da FC (HECTOR; FREY; HARTL, 2016; MAHENTHIRALINGAM, 2014; PARKINS; FLOTO, 2015; SALSGIVER et al., 2016). Muitos destes são bacilos gram-negativos não fermentadores da glicose (BGNNF), entre os quais estão bactérias do CBc, *Burkholderia gladioli*, *A. xylosoxidans*, *S. maltophilia*, *Ralstonia mannitolytica* e *Pandoraea* spp, *Inquilinus* spp e *Cupriavidus* spp. (MAHENTHIRALINGAM, 2014; PARKINS; FLOTO, 2015). Outros emergentes, fora do grupo de BGNNF, são MRSA, fungos, anaeróbios e micobactérias não tuberculosas.

Diversos fatores podem estar contribuindo para estas mudanças epidemiológicas na microbiologia da FC, incluindo maior sobrevida dos pacientes, aprimoramento das técnicas de

identificação microbiológica, pressão seletiva dos agentes antimicrobianos, medidas de controle de infecção e fatores ambientais, dentre outros (LIPUMA, 2010; SALSGIVER et al., 2016).

Laboratório de microbiologia clínica habilitado em FC é primordial para o cuidado de indivíduos com esta doença. A detecção e a identificação apropriadas dos microrganismos, assim como os testes de sensibilidade antimicrobiana, requerem técnicas microbiológicas específicas e são fundamentais para o abordagem destas infecções (SAIMAN et al., 2014)

Historicamente, por se tratar de infecções polimicrobianas, a identificação microbiológica na FC tem sido baseada em culturas realizadas em meios seletivos, com o objetivo de favorecer o crescimento e a identificação de microrganismos de interesse. As atenções se voltam especialmente para a detecção de gram-negativos aeróbios e *S. aureus.* Nessas culturas, os bacilos gram-negativos emergentes podem ser confundidos com *P. aeruginosa* ou CBc ou simplesmente relatados como BGNNF, sem especificações. Fenótipos diferentes de colônias bacterianas podem coexistir na cultura, como *P. aeruginosa* mucoide e *S. aureus* com crescimento na forma de colônias puntiformes (SCV: *small collony variant*), também são desafios que dependem da *expertise* de profissionais para correta identificação. Além das culturas em meios seletivos, testes fenotípicos extensivos e testes moleculares são necessários. Recursos humanos e tecnológicos que atendam a esta demanda costumam estar disponíveis apenas em laboratórios especializados em FC. É fundamental também que, durante a solicitação para a realização das culturas, o médico especifique que o espécime clínico é de indivíduo com diagnóstico ou suspeita de FC para que o processamento da mesma siga todas as etapas necessárias e especificas (PARKINS; FLOTO, 2015; MARQUES, 2011)

Métodos moleculares são recomendados para caracterização das espécies de *Achromobacter*, CBc e dos gêneros *Ralstonia*, *Cupriavidus* e *Pandoraea* (GBEFC, 2016). Diferentes tecnologias tem sido incorporadas à rotina dos laboratórios, a maioria baseada em amplificação do PCR, como as que têm como alvo os genes *rec*A e 16S rRNA. O desenvolvimento e a incorporação de tecnologias de diagnóstico molecular vêm tendo, e ainda terão, grande impacto nas mudanças epidemiológicas relacionadas à microbiologia da FC (PARKINS; FLOTO, 2015). A identificação da microbiota cada vez mais diversa é hoje uma realidade que exige a aproximação do médico e do microbiologista para que, através de

pesquisas translacionais, promova-se melhor entendimento acerca da importância destes microrganismos emergentes e defina-se medidas necessárias para o cuidado dos pacientes.

1.4 ***Achromobacter xylosoxidans***

Bactérias do gênero *Achromobacter* são amplamente distribuídas no ambiente, preferencialmente em meios aquáticos e no solo (AMOUREUX et al., 2013b). São BGNNF classificados como aeróbios, mas algumas vezes sobrevivem também em meios anaeróbios. A presença de flagelos peritríqueos longos conferem ao *Achromobacter* spp. mobilidade tipo *swimming* altamente eficaz. Pouco se conhece sobre os mecanismos de virulência e adaptação do *Achromobacter* spp, porém estudos com *P. aeruginosa* na FC sugerem que os fenótipos que conferem mobilidade (tipo *swimming*, *swarming* e *twitching*) e produção de toxinas sejam importantes para a infecção aguda, enquanto a produção de biofilme e a redução de fatores de virulência sejam mecanismos de adaptação que favorecem a cronicidade da infecção (RASHID; KORNBERG, 2000; WINSTANLEY; O'BRIEN; BROCKHURST, 2016). Outra característica marcante do *Achromobacter* sp é a alta resistência intrínseca a antimicrobianos (SWENSON; SADIKOT, 2015).

Em humanos, muitos membros do gênero não são patogênicos e podem fazer parte da flora saprófita em ouvidos, intestinos e pulmões (SWENSON; SADIKOT, 2015). Algumas espécies, principalmente *A. xylosoxidans,* são encontradas em infecções nosocomiais. No ambiente hospitalar, podem formar nichos em respiradores, nebulizadores, ou contaminar diferentes soluções consideradas estéreis, como soluções salinas, fluidos para administração intravenosa, soluções de diálise, gel para ultrassonografia e soluções antissépticas, como o clorexidine (REVERDY et al., 1984). Outras vezes, foram identificadas causando pneumonia (LIU et al., 2016), bacteremia (DUGGAN et al., 1996; TUREL et al., 2013), endocardite (TOKUYASU et al., 2012), infecção urinária (TENA et al., 2008), meningite (SEPKOWITZ; BOSTIC; MASLOW, 1987), entre outras infecções. Doenças de base como imunodeficiência (BELLISSIMO et al., 2014), doenças hematológicas (ADAM et al., 2014; AISENBERG; ROLSTON; SAFDAR, 2004), insuficiência renal (CHANDRASEKAR; ARATHOON; LEVINE, 1986) e, principalmente, FC, entre outras, favorecem a infecção por *Achromobacter* spp (SWENSON; SADIKOT, 2015).

A nomenclatura do gênero já sofreu modificações algumas vezes. Inicialmente foi denominado *Achromobacter*, depois *Alcaligenes* e, mais tarde, novamente *Achromobacter*. Com base em análises de sequenciamento genético, sabe-se atualmente que as bactérias do gênero *Achromobacter* se assemelham mais às do gênero *Bordetella* do que às do *Acaligenes* e suspeita-se que esses dois tenham um antecessor comum (SWENSON; SADIKOT, 2015).

A descrição da espécie *A. xylosoxidans* se deu em 1971, por Yabucchi e Oyama, após isolamento em pacientes com otorreia. Até então, o gênero *Achromobacter* contava com cinco espécies descritas por outros autores (YABUUCHI; OHYAMA, 1971). A complexidade deste gênero é muito grande e ainda hoje sofre mudanças frequentes. A caracterização das espécies tem sido possível nos últimos anos graças a métodos moleculares mais modernos, como *multilocus sequence typing* (MLST) (AMOUREUX et al., 2016; GOMILA et al., 2014; RIDDERBERG; WANG; NØRSKOV-LAURITSEN, 2012; SPILKER; VANDAMME; LIPUMA, 2012). Atualmente há 16 espécies e duas subespécies de *Achromobacter* descritas (LSPN, 2016). Antes da descrição das novas espécies, a maioria dos estudos se referia ao gênero *Achromobacter* spp como *A. xylosoxidans* porque os métodos convencionais de identificação o classificavam desta forma, sem poder suficiente para distinguir as espécies (AMOUREUX et al., 2016). Consequentemente, a epidemiologia e o impacto clínico das espécies de *Achromobacter* na FC ainda são confusos e carecem de muitos esclarecimentos. Porém, os estudos mais recentes ratificaram a espécie *A. xylosoxidans* como a mais frequente nesta população, seguida da *A. rhulandii*. Respeitando a denominação original dos estudos e a nomenclatura usada nos registros de FC (CYSTIC FIBROSIS FOUNDATION, 2015; GBEFC, 2015), usaremos, a partir de agora, o termo *A. xylosoxidans* em referência ao gênero *Achromobacter*, com outras especificações quando necessário.

Na FC a primeira descrição de *A. xylosoxidans* se deu na década de 1980 em estudo visando a identificação de BGNNF emergentes (KLINGER; THOMASSEN, 1985). Desde então, a prevalência deste microrganismo vem aumentando em diferentes centros de FC do mundo (LIPUMA, 2010; MAHENTHIRALINGAM, 2014; PARKINS; FLOTO, 2015). A faixa de prevalência descrita na literatura é ampla, variando entre 1,1 e 29,3%; (BURNS et al., 1998; COOLS et al., 2016; KLINGER; THOMASSEN, 1985b; KRZEWINSKI et al., 2001; MOISSENET et al., 1997; PEREIRA et al., 2011; RASO et al., 2008; STEINKAMP et al., 2005). Os trabalhos que descrevem os valores maiores, em geral, se referem à prevalência cumulativa. De acordo com o Registro Americano de FC de 2014, a prevalência de *A* .

xylosoxidans, baseada na presença de pelo menos uma cultura positiva por ano, vem crescendo gradualmente de 1996 a 2014, tendo sido encontrada em torno de 6-7% dos pacientes no último registro (CYSTIC FIBROSIS FOUNDATION, 2015). No Registro Brasileiro de Fibrose Cística (REBRAFC), esta frequência variou entre 2,1 e 3% no período de 2009 a 2014; a prevalência maior também foi em 2014 (GBEFC, 2015). No entanto, estas frequências registradas provavelmente são subestimadas devido à frequente identificação equivocada deste patógeno como *P. aeruginosa*, *S. maltophila* e bactérias do CBc, principalmente em laboratórios com técnicas automatizadas ou não especializados em FC (SAIMAN et al., 2001).

A. xylosoxidans pode ser detectado esporadicamente ou causar infecção crônica. Na FC, a associação com infecção crônica por *P. aeruginosa* é comum e alguns estudos sugerem que o tratamento específico para este agente seja fator de risco para a infecção por *A. xylosoxidans* (KANELLOPOULOU et al., 2004; LAMBIASE et al., 2011). Cepas diferentes de *A. xylosoxidans* podem colonizar um mesmo paciente simultaneamente ou em momentos distintos. Já na cronicidade uma única cepa costuma estar envolvida (AMOUREUX et al, 2013a; RIDDEBERG et al., 2011).

Além da resistência intrínseca a muitos antibióticos, outras preocupações em relação a este patógeno já documentadas na FC são a transmissibilidade (LAMBIASE et al., 2011), inclusive com detecção recente de cepas epidêmicas na Bélgica (COOLS et al., 2016) e a associação com aumento da inflamação pulmonar (HANSEN et al., 2010). A prevalência deste microrganismo tem sido maior em indivíduos com comprometimento pulmonar mais grave e com exacerbações mais frequentes da FC (DE BAETS et al., 2007). Um estudo de caso controle retrospectivo realizado na Dinamarca conseguiu demostrar queda significativa da função pulmonar em pacientes com FC infectados por *A. xylosoxidans* que desenvolveram anticorpos específicos (RØNNE HANSEN et al., 2006), enquanto a maioria dos outros não conseguiu associar este microrganismos com deterioração clínica na FC (DE BAETS et al., 2007; LAMBIASE et al., 2011; RASO et al., 2008; TAN et al., 2002).

Até o momento não há nenhuma recomendação terapêutica específica para tentativa de erradicação nem para tratamento da infecção crônica por *A. xylosoxidans*. Uma série de medidas de prevenção e controle de infecções na FC são primordiais, uma vez que os indivíduos apresentam colonizações polimicrobianas e que cada agente tem particularidades individuais (SAIMAN et al., 2014).

Não há testes laboratoriais de suscetibilidade antimicrobiana padronizados para este microrganismo. Um centro de referência na Dinamarca vem usando amoxicilina com clavulanato sistêmico associado com colestimetado inalatório na tentativa de erradicação do *A. xylosoxidans* (RØNNE HANSEN et al., 2006), mas não há evidência ainda para fazer desta medida uma recomendação. Para o tratamento de exacerbações da FC em que este patógeno possa estar envolvido, algumas opções são sugeridas. Este microrganismo costuma apresentar resistência intrínseca a alguns antibióticos usados para tratar *P. aeruginosa*, como aminoglicosídeos e ciprofloxacin (CHMIEL et al., 2014). Sugere-se, então, a associação de pelo menos dois antibióticos. Meropenem ou imipenem associados com sulfametoxazol-trimetoprim, ciprofloxacin ou minocilina são opções terapêuticas recomendadas pela American Throracic Society (CHMIEL et al., 2014). Um consenso europeu, publicado em 2012, incluiu, além destas, outras alternativas como piperacilina com tazobactam, ticarcilina com ácido clavulânico, aztreonam e ceftazidime, entre outros, também em esquemas combinados (DÖRING et al., 2012). Estes documentos podem ser referências usadas quando necessário.

Esclarecimentos a respeito do ainda controverso impacto clínico do *A. xylosoxidans* na FC são importantes para que que possam ser desenvolvidas estratégias de abordagem adequadas.

2 **JUSTIFICATIVA**

Trabalho recente realizado no Rio de Janeiro (PEREIRA et al., 2011) evidenciou frequência elevada de *A. xylosoxidans* (21% de 39 indivíduos) em pacientes com FC, acometendo predominantemente crianças, o que difere de outras publicações em que o predomínio foi entre adolescentes e adultos (DE BAETS et al., 2007; HAUSER et al., 2011). Foi questionado se este resultado estaria relacionado à gravidade da FC nesta população e levantada a hipótese da infecção pelo *A. xylosoxidans* não ser inócua para estes indivíduos. A necessidade destes esclarecimentos motivou este estudo, com a intenção de que as conclusões possam auxiliar na tomada de decisões clínicas e contribuir para o cuidado de indivíduos com FC.

3 **OBJETIVOS**

3.1 **Objetivo geral**

Avaliar o impacto clínico da infecção por *A. xylosoxidans* em pacientes com FC acompanhados no Rio de Janeiro, no que se refere à função pulmonar, índice de massa corporal (IMC) e frequência de exacerbações no momento da infecção e dois anos após.

3.2 **Objetivos Específicos**

3.2.1 - Identificar a prevalência de *A. xylosoxidans* numa coorte retrospectiva de indivíduos com FC acompanhados no Rio de Janeiro.

3.2.2 - Comparar três grupos (infectados crônicos, intermitentes e nunca infectados por *A. xylosoxidans*; todos com coinfecção crônica por *P. aeruginosa*) em relação a:

a. Características demográficas da população, critérios diagnósticos de FC, presença de insuficiência pancreática, diabetes relacionado à FC, doença hepática e quanto à presença ou não da mutação F508del em seu genótipo.

b. Coinfecções crônicas apresentadas.

c. Volume expiratório forçado no primeiro segundo (VEF_1), capacidade vital forçada (CVF), IMC e número médio de internações anuais em dois momentos: no momento da infecção pelo *A. xylosoxidans* (M1) e dois anos após (M2).

4 MATERIAIS E MÉTODOS

4.1 Aspectos éticos

O projeto deste estudo foi registrado na Plataforma Brasil e aprovado pelos comitês de ética em pesquisa das duas instituições envolvidas, sob os registros CAAE:00716512.0.0000.5259 e 00716512.0.3001.5269 (ANEXOS I e II, respectivamente). O termo de consentimento livre e esclarecido (TCLE) foi dispensado mediante justificativa de ser trabalho retrospectivo e sem intervenção, com alguns indivíduos da população já falecidos. Garantiu-se o sigilo de informações de identificação pessoal.

4.2 Locais do estudo

Este estudo foi realizado Hospital Universitário Pedro Ernesto (HUPE) /Universidade do Estado do Rio de Janeiro (UERJ) e no Instituto Nacional de Saúde da Mulher, da Criança e do Adolescente Fernandes Figueira (IFF)/Fundação Oswaldo Cruz (Fiocruz). Estas instituições são Centros de Referência para FC no Estado do Rio de Janeiro. Crianças e adolescentes são acompanhados no IFF, enquanto adultos (pacientes com 18 anos ou mais) fazem o seguimento na UERJ.

A rotina assistencial é semelhante em ambos os centros, com consultas realizadas idealmente em intervalos de até três meses. Nas consultas, os pacientes passam por avaliação clínica, em equipe multiprofissional e são realizadas culturas de secreção respiratória (escarro ou, no caso de crianças não expectorantes, *swab* de orofaringe) e espirometria. Este último exame, nas crianças, é conseguido habitualmente naquelas com seis anos de idade ou mais, na dependência da capacidade cognitiva de realizar o teste. As culturas de secreção respiratória de ambos os centros são realizadas no Laboratório de Bacteriologia (LBACT) do HUPE/UERJ, seguindo protocolos padronizados estabelecidos para culturas em pacientes com FC (GILLIGAN; KISKA; APPLEMAN, 2006). A identificação final dos microrganismos por métodos moleculares é realizada no Laboratório 2 da Disciplina de Microbiologia da Faculdade de Ciências Médicas da UERJ.

4.3 **Desenho experimental**

Este foi um estudo retrospectivo, tipo caso-controle, com portadores de FC.

4.4 **População do estudo**

Com base nos registros do LBACT do HUPE/UERJ, foi feito um levantamento dos pacientes com FC regularmente acompanhados no HUPE/UERJ e no IFF/Fiocruz que apresentaram pelo menos uma cultura de amostra respiratória positiva para *A. xylosoxidans* no período entre janeiro de 2003 e dezembro de 2011.

Posteriormente, foram selecionados, deste grupo, aqueles que também preenchiam critérios de infecção crônica por *P. aeruginosa,* ou seja, mais de 50% das culturas positivas para este microrganismo em um ano (LEE et al., 2003), com o mínimo de quatro coletas por ano com um mês ou mais de intervalo entre elas.

Usando este mesmo critério de cronicidade, esta população foi dividida em dois grupos:

- Grupo I: infectados crônicos por *A. xylosoxidans*.
- Grupo II: infectados intermitentes por *A. xylosoxidans*. Foram considerados intermitentes aqueles com menos de 50% das culturas positivas.

Um terceiro grupo (Grupo III), foi selecionado nos registros do LBACT, composto por indivíduos também colonizados crônicos por *P. aeruginosa*, mas que nunca tiveram culturas positivas para *A.xylosoxidans,* pareados com outros dos grupos I e II de acordo com idade (±1 ano) e sexo.

4.4.1 <u>Critérios de inclusão</u>

A. Diagnóstico confirmado de FC de acordo com os critérios da *Cystic Fibrosis Foundation* (FARRELL et al., 2008; ROSENSTEIN; CUTTING, 1998).

B. Acompanhamento regular no período de 2003 a 2011, com coleta mínima de quatro culturas de secreção respiratória por ano, com intervalo mínimo de um mês entre elas.

C. Ter infecção crônica por *P. aeruginosa* (LEE et al., 2003).

4.4.2 Critérios de exclusão

A. Infecção crônica por bactérias do CBc, com base no mesmo critério utilizado para os outros microrganismos.

Falta de acesso a documentos fonte para a coleta de dados (resultados das culturas ou prontuários médicos).

4.5 **Métodos microbiológicos**

Para identificação do *A. xylosoxidans*, as amostras de secreção respiratória foram caracterizadas inicialmente por provas fenotípicas, como BGNNF ou *Achromobacter* spp. pelo LBACT. A seguir, foram encaminhadas para o Laboratório 2 da Disciplina de Microbiologia e Imunologia - UERJ e estocadas em solução de leite desnatado a 10% (Skim Milk, Difco Laboratories, Detroit, MI, EUA) contendo glicerol a 10% e mantidas a -20°C até a realização dos testes moleculares.

Para identificação molecular, o DNA de cada *Achromobacter* isolado foi extraído por método de lise por ebulição e todo o gene 16S rRNA foi amplificado por PCR, sequenciado e identificado pelo programa BLAST em confrontação com database GenBank. A presença do gene $bla_{\text{oxa-114-like}}$, especifico de *Achromobacter xylosoxidans* foi investigada por PCR como descrito por Turton et al., 2011. Após amplificação, os produtos de PCR foram sequenciados e comparados com sequências depositadas no banco de dados GenBank usando o programa BLAST.

4.6 **Coleta de dados**

4.6.1 Fontes de dados

Foi criada uma planilha específica para este estudo no intuito de registrar os dados clínicos e bacteriológicos. Os documentos fonte foram os prontuários médicos e os resultados de culturas respiratórias armazenados no LBACT/ HUPE/UERJ.

4.6.2 Descrição da população

A população geral do estudo foi descrita de acordo com dados demográficos (idade, sexo), critérios diagnósticos de FC e existência de insuficiência pancreática exócrina, DRFC e hepatopatia. Terapia de reposição exógena de enzimas pancreáticas foi considerada indicador de insuficiência pancreática exócrina. A existência de DRFC e de hepatopatia foram baseadas no registro médico destes diagnósticos nos prontuários.

4.6.3 Coinfecções crônicas

Foram descritas com base nos mesmos critérios de cronicidade usados para os outros microrganismos (LEE et al., 2003).

4.6.4 Desfechos clínicos

Com o objetivo de avaliar o impacto clínico, os três grupos foram comparados quanto a dados transversais em dois momentos:

- Momento 1 (M1): o da infecção pelo *A. xylosoxidans* (para os grupos I e II) ou o momento do pareamento (grupo III).
- Momento 2 (M2): o mais próximo possível de 24 meses depois do M1.

Em relação aos dados de função pulmonar, foram registrados o volume expiratório forçado no primeiro segundo (VEF_1) e a capacidade vital forçada (CVF), em valores percentuais do predito para idade, sexo e altura, de todos os pacientes com idade e capacidade cognitiva para terem realizado este exame. Foram selecionados os melhores valores de VEF_1 e CVF que eram mais próximos do M1 e do M2. As medidas foram obtidas no equipamento de função pulmonar modelo HD CPL (nSpire Health, Inc, USA), seguindo a padronização recomendada pela American Thoracic Society (MILLER; GILLIGAN, 2003). Os resultados foram baseados nos valores preditos da função pulmonar para população brasileira (MALLOZI, 1996; PEREIRA,2007).

O peso e a altura dos pacientes foram usados para calcular o índice de massa corporal (IMC), que corresponde ao peso (em kg) dividido pelo quadrado da altura (em metros). Esta variável foi utilizada em seu número absoluto, uma vez que o desfecho de interesse era a variação do M1 para o M2 e que o grupo estudado incluía crianças, adolescentes e adultos, grupos em que a classificação do estado nutricional é diferente. Para crianças e adolescentes, a classificação do estado nutricional se baseia em percentis e escore Z do IMC para idade e sexo, enquanto para adultos é feita por faixas categóricas do valor do IMC (TURCK et al., 2016).

O número exacerbações respiratórias da FC foi outra variável de interesse, aferida como frequência de média de internações anuais do M1 ao M2.

4.7 **Metodologia estatística**

Os dados numéricos foram apresentados como médias ± desvio padrão (SD) ou medianas e intervalos (mínimo-máximo). Os dados categóricos foram apresentados como frequências (%). As variáveis apresentaram distribuição não-normal de acordo com o teste de Kolmogorov-Smirnov e, por isso, foram aplicados testes não-paramétricos. Kruskal-Wallis ANOVA, com o teste de comparações múltiplas de Dunn correspondente, foi utilizado para comparar as variáveis numéricas entre os três grupos. O teste exato de Fisher foi utilizado para comparar as variáveis categóricas. Quando a associação entre variáveis categóricas dentro do grupo foi significativa a 5%, o teste exato de Fisher, definido para cada grupo de pares separadamente, foi usado. Por isso, procurou-se identificar quais os grupos diferiam entre si a um nível de 1,7%. Um nível de 1,7% (5% dividido pelas comparações Número: 0,05 / 3 = 0,017) foi utilizado para controlar a erro do tipo I. Para determinar a existência de variações significativas nos valores VEF_1, CVF e IMC entre M1 e M2, o teste de Wilcoxon foi utilizado. A análise dos dados foi realizada utilizando o software SAS versão 6.11 (SAS Institute, Inc., EUA). O nível de significância estatística foi estabelecido em $P<0.05$.

5 **RESULTADOS**

Esta tese resultou em dois artigos científicos até o momento.

O primeiro, com os dados principais do estudo, foi publicado no *Brazilian Journal of Medical and Biological Research*, em 2016, e encontra-se aqui em Word e, no APÊNDICE I, na forma como foi publicado.

O segundo, motivado por questionamentos que surgiram durante o primeiro estudo, foi recentemente submetido para publicação no *BMC Pulmonary Medicine* e a confirmação da submissão encontra-se no APÊNDICE II. Trata-se de um relato de caso de dois irmãos portadores de FC colonizados por *A. xylosoxidans* por mais de 10 anos e com curso clínico de gravidades diferentes. Como tinham as mesmas mutações genéticas e viviam no mesmo ambiente, foram considerados promissores para identificarmos se havia alguma diferença entre mecanismos de virulência e de adaptação do *A. xylosoxidans* em cada um deles que pudessem contribuir para esta diferença. Foi obtido consentimento assinado para a publicação destes relatos.

5.1 **Artigo 1**

Title: What is the clinical impact of *Achromobacter xylosoxidans* colonization/infection in patients with cystic fibrosis?

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Running title: *Achromobacter xylosoxidans* in cystic fibrosis

Abstract

The rate of diagnosis of colonization/infection of the airways with *Achromobacter xylosoxidans* has increased in cystic fibrosis patients; however, its clinical significance is still controversial. This retrospective case-control study aimed to evaluate the clinical impact of *A. xylosoxidans* colonization/infection in cystic fibrosis patients. Individuals chronically colonized/infected (n=10), intermittently colonized/infected (n=15), and never colonized/infected with *A. xylosoxidans* (n=18) were retrospectively evaluated during two periods that were two years apart. The demographic characteristics, clinical data, lung

function, and chronic bacterial co-colonization data were evaluated. Of the total study population, 87% were pediatric patients and 65.1% were female. Individuals chronically colonized/infected with *A. xylosoxidans* had decreased forced expiratory volume in one second (51.7% in the chronic colonization/infection group vs. 82.7% in the intermittent colonization/infection group vs. 76% in the never colonized/infected group). The rate of co-colonization with methicillin-resistant *Staphylococcus aureus* was higher in individuals chronically colonized/infected with *A. xylosoxidans* (P=0.002). The changes in lung function over two years in the three groups were not significant, although a trend toward a greater decrease in lung function was observed in the chronically colonized/infected group. There was a greater number of annual hospitalizations in patients chronically colonized/infected with *A. xylosoxidans* (P=0.033). In cystic fibrosis patients, an increased frequency of *A. xylosoxidans* colonization/infection was present among children, and reduced lung function in patients chronically colonized/infected with *A. xylosoxidans* was observed. In addition, no differences in clinical outcomes during the two year period, except for an increased number of hospitalizations in patients with *A. xylosoxidans,* were observed.

Introduction

The genus *Achromobacter* contains genetically distinct species and subspecies and has not been fully characterized (1-5). *Achromobacter* species are Gram-negative, aerobic, nonfermenters of glucose bacilli that are widely distributed in the environment. *A. xylosoxidans* is the most common bacillus in clinical samples and is recognized as an emerging and multidrug-resistant microorganism that causes various opportunistic infections and nosocomial outbreaks (3,6). Most knowledge about *A. xylosoxidans* has been obtained from studies on populations living in regions where cystic fibrosis (CF) is more prevalent (3,6).

The rate of colonization/infection with *A. xylosoxidans* reported in individuals with CF varies between 2% and 17.9% (7,8) and is increasing worldwide. However, this frequency may be underestimated because this organism can be confused with *Pseudomonas aeruginosa*, bacteria from the *Burkholderia cepacia* complex (BCc), and *Stenotrophomonas maltophilia*, particularly in laboratories that are not specialized for CF evaluation (9).

The factors that predispose patients to colonization/infection have not been fully elucidated. It is speculated that frequent exposure to antibiotics, particularly during treatment

for chronic colonization with *P. aeruginosa*, may favor the emergence of this and other Gram-negative, multidrug-resistant bacteria (10,11). The possibility of person-to-person transmission, the association of *A. xylosoxidans* colonization/infection with pulmonary inflammation, and an increased frequency of exacerbations have been demonstrated; however, the clinical impact of colonization/infection in CF patients is still controversial (6,11-15). Therefore, the present study aimed to evaluate the clinical impact of *A. xylosoxidans* colonization/infection in patients with CF.

Material and Methods

Study Design

This retrospective case-control study evaluated patients with a confirmed diagnosis of CF (16) who were regularly monitored at the Instituto Nacional Fernandes Figueira of the Fundação Oswaldo Cruz (IFF-Fiocruz) and Policlínica Piquet Carneiro of the Universidade do Estado do Rio de Janeiro (PPC-UERJ) and whose respiratory secretion culture results obtained between January 2003 and December 2011 were available at the Laboratório de Bacteriologia of the Hospital Universitário Pedro Ernesto (LBACT-UERJ).

The protocol conformed to the World Medical Association Declaration of Helsinki and was approved by the Research Ethics Committee of the UERJ under number CAAE: 00716512.0.3001.5269.

Patients

A total of 238 individuals with CF were regularly monitored in these referral centers, of whom 25% were adults (≥18 years). The routine follow-up period consisted of quarterly consultations, except for infants, who were monitored monthly. The interval between consultations was shortened depending on the clinical need. At each visit, the general medical condition, weight, height, and lung function of patients were evaluated, and respiratory secretions were obtained for culture (sputum or oropharyngeal swab for non-expectorating children). All material obtained at these centers was sent to the LBACT-UERJ, where cultures of respiratory secretions were conducted according to standardized protocols established for CF patients; cultures were performed every three months throughout the study (17).

Identification of *Achromobacter*

All *A. xylosoxidans* isolates were identified using both phenotypic and molecular methods.

Phenotypic methods. Isolates identified as *Achromobacter* spp. by the Vitek 2 Compact system using Gram-negative (GN) cards (reference no. 21341; bioMérieux) were subjected to further identification via a large panel of phenotypic tests, as previously described (18,19).

Molecular methods. To identify each isolate, DNA was extracted by the boiling lysis method, and the entire 16S rRNA gene was amplified by PCR, sequenced, and used for BLAST searches against the GenBank database (20). The presence of the *A. xylosoxidans* species-specific marker *bla*OXA-114 was investigated by PCR amplification as described by Turton et al. (6). After amplification, the PCR products were sequenced and compared with sequences in the GenBank database at the NCBI using BLAST.

Inclusion and exclusion criteria

The respiratory secretion culture results of patients with CF were evaluated using the LBACT-UERJ database. The inclusion criteria were as follows: 1) patients with one or more cultures positive for *A. xylosoxidans* (the term 'colonization/infection' is used in reference to positive cultures); and 2) patients colonized/infected with *A. xylosoxidans* and chronically colonized with *P. aeruginosa*, defined as more than 50% of cultures positive for the latter agent during one year (21). The exclusion criteria consisted of colonization with BCC bacteria and/or the absence of chronic colonization with *P. aeruginosa*.

Definition of the groups

The group was subdivided according to their *A. xylosoxidans* colonization/infection status into a chronically colonized/infected group and an intermittently colonized/infected group. The criterion for chronic colonization/infection by *A. xylosoxidans* was the same as that adopted for *P. aeruginosa* (21). Any shorter frequency was considered to be intermittent colonization/infection. The control group consisted of individuals who never had a positive culture for *A. xylosoxidans*, and subjects were matched with those in the case groups according to age (± 1 year), gender, and chronic colonization with *P. aeruginosa*. All patients were chronically colonized by *P. aeruginosa*, and the status of *A. xylosoxidans* (chronic, intermittent, and never) was defined as described previously. Thus, the three study groups were as follows: group I, chronic colonization/infection with *A. xylosoxidans* and chronic colonization with *P. aeruginosa*; group II, intermittent colonization/infection with *A.*

xylosoxidans and chronic colonization with *P. aeruginosa*; and group III, never colonized/infected with *A. xylosoxidans* but chronically colonized by *P. aeruginosa*.

Clinical outcomes

The general population was described according to the demographic characteristics, diagnostic criteria for CF, and the presence of exocrine pancreatic insufficiency, cystic fibrosis-related diabetes, and liver disease. The frequency of the F508del mutation was described when available. In addition, other chronic bacterial co-colonizations were recorded by adopting the same criteria for chronic colonization as that used for *P. aeruginosa* (21).

The cross-sectional registration of clinical data was performed on two occasions: when the first positive culture for *A. xylosoxidans* occurred (moment 1 – M1) and as close as possible to 24 months after the first positive culture (moment 2 – M2). In the control group, data from M1 was paired with that of subjects in the case groups (groups 1 and 2), and the same criteria were followed for M2.

Regarding lung function, the values of forced expiratory volume in one second (FEV_1) and forced vital capacity (FVC) were recorded for all patients old enough to perform these tests. We recorded the best lung function value that was closest to the time of initial colonization; similarly, we also recorded the best lung function that was obtained closest to 24 months later. The measurements were obtained with an HD CPL (nSpire Health, Inc., Longmont, CO, USA) following the appropriate standards set by the American Thoracic Society (22). The pulmonary function results are expressed as a percentage of the predicted values for the Brazilian population (23). The weight and height of patients were used to calculate the body mass index (BMI). The FEV_1, FVC, and BMI were compared for the two time periods both within and between groups. The median number of annual admissions was also compared between the groups.

Statistical analysis

Numerical data are expressed as the mean ± SD or medians and ranges (minimum-maximum), whereas categorical data are expressed as frequencies (%). The variables had a non-normal distribution according to the Kolmogorov-Smirnov test; therefore, non-parametric tests were applied. Kruskal-Wallis ANOVA, with the corresponding Dunn’s multiple comparison test, was used to compare the numerical variables between the three groups. Fisher’s exact test was used to compare categorical variables. When the association between categorical variables within the group was significant at 5%, Fisher's exact test, set for each

peer group separately, was used. Thus, we aimed to identify which groups differed from each other at a level of 1.7% [a level of 1.7% (5% divided by the number comparisons: 0.05 / 3 = 0.017) was used to control the Type I error]. To verify the existence of significant variations in FEV_1, FVC, and BMI values between M1 and M2, the Wilcoxon signed rank test was used. Data analysis was performed using SAS software version 6.11 (SAS Institute, Inc., Cary, NC, USA). The level of statistical significance was set at $P<0.05$.

Results

Of the 238 individuals with culture results, 47 (19.7%) had at least one positive culture for *A. xylosoxidans*, among whom 25 met the inclusion criteria for the study. Ten patients were classified as chronically colonized/infected, and 15 were classified as intermittently colonized/infected. The control group consisted of 18 patients. No participants died during the study period. The general characteristics of the study population and a comparison between groups at baseline are shown in Table 1.

The median period of chronic colonization with *P. aeruginosa* was one year, and the range varied from one to three years. The baseline values for age, gender, F508del mutation frequency, exocrine pancreatic insufficiency, diabetes, liver disease, length of colonization with *P. aeruginosa*, and BMI were similar between the three groups. The FEV_1 and FVC values were lower in the group chronically colonized/infected with *A. xylosoxidans*, but this difference was not significant compared with the other groups (Table 1).

Table 1. General characteristics of the study population and comparison between groups at baseline.

Variables	Total sample		Chronic colonization/infection with *A. xylosoxidans* (Group I)		Intermittent colonization/infection with *A. xylosoxidans* (Group II)		Never colonized/infected with *A. xylosoxidans* (Group III)		P value
	N		n		N		n		
Age (years)	43	7 (1–37)	10	10.5 (3–18)	15	7 (2–33)	18	7.5 (1–37)	0.64
Gender (female)	43	28 (65.1)	10	5 (50)	15	9 (60)	18	14 (77.8)	0.29
Family history	43	4 (9.3)	10	2 (20)	15	1 (6.7)	18	1 (5.6)	0.43
Neonatal screening	43	5 (11.6)	10	1 (10)	15	1 (6.7)	18	3 (16.7)	0.83
Homozygous F508del frequency	37	9 (24.3)	8	0 (0)	13	5 (38.5)	16	4 (25)	0.14
Heterozygous F508del frequency	37	16 (43.2)	8	3 (37.5)	13	7 (53.9)	16	6 (37.5)	0.69
Another mutation / mutation unidentified	37	12 (32.4)	8	5 (62.5)	13	1 (7.7)	16	6 (37.5)	0.027*
Exocrine pancreatic insufficiency	43	38 (88.4)	10	10 (100)	15	14 (93.3)	18	14 (77.8)	0.26
Cystic fibrosis-related diabetes	43	2 (4.6)	10	1 (10)	15	1 (6.7)	18	0 (0)	0.72
Liver disease	43	1 (2.3)	10	0 (0)	15	1 (6.7)	18	0 (0)	0.78
Chronic *P. aeruginosa* time (years)	43	1 (1–3)	10	1.5 (1–3)	15	1 (1–2)	18	1 (1–3)	0.23
FEV_1–M1	24	70.1 (27.3–112)	6	51.7 (27.3–95.1)	6	82.7 (55.1–112)	12	76 (35–108.5)	0.15
FVC–M1	24	86.4 (41.9–115)	6	67.8 (41.9–90.7)	6	98.7 (76.1–110)	12	82.7 (49.9–115)	0.09
BMI–M1	43	15.9 (11.5–27.1)	10	15.4 (14.1–23.4)	15	16.2 (13.6–22.9)	18	16.4 (11.5–27.1)	0.31

Results expressed as median and ranges (minimum–maximum) or number (%).

FEV_1–M1: forced expiratory volume in one second at baseline; FVC–M1: forced vital capacity at baseline; BMI–M1: body mass index at baseline.

*Group I # Group II (Fisher' exact test set for each peer group separately).

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When the two periods (M1 and M2) were compared, significant differences were observed both in the FEV_1 (P=0.014) and the FVC (P=0.016) for the total sample but not for the patient groups. The median number of annual admissions during the study period was significantly different between groups (P=0.033). Information regarding the pulmonary function, BMI, and clinical data for each group at M1 and M2 are presented in Table 2.

Table 2. Lung function, body mass index, and clinical data according to the groups at baseline and after 24 months.

Variable	Group	N	Baseline	After 24 months	P valor
FEV_1 (% predicted)	Chronic colonization/infection with *A. xylosoxidans* (Group I)	6	51.7 (27.3–95.1)	45.3 (16.6–88.7)	0.063
	Intermittent colonization/infection with *A. xylosoxidans* (Group II)	6	82.7 (55.1–112)	78.9 (40.2–125.3)	0.15
	Never colonized/infected with *A. xylosoxidans* (Group III)	12	76.0 (35–108.5)	82.7 (24–102.7)	0.30
	Total	24	70.1 (35–112)	75.5 (16.6–125.3)	**0.014**
FVC (% predicted)	Chronic colonization/infection with *A. xylosoxidans* (Group I)	6	67.8 (55–90.7)	69.5 (27.5–92.5)	0.44
	Intermittent colonization/infection with *A. xylosoxidans* (Group II)	6	98.7 (76.1–110)	92.1 (61.4–117.5)	0.22
	Never colonized/infected with *A. xylosoxidans* (Group III)	12	82.7 (35–108.5)	84.1 (25.6–102.5)	0.061
	Total	24	86.4 (35–110)	84.4 (25.6–117.5)	**0.016**
BMI (kg/m^2)	Chronic colonization/infection with *A. xylosoxidans* (Group I)	10	15.4 (14.1–23.4)	16.6 (14.1–23.1)	0.94
	Intermittent colonization/infection with *A. xylosoxidans* (Group II)	15	16.2 (14.2–22.9)	16.4 (13.6–22.9)	0.67
	Never colonized/infected with *A. xylosoxidans* (Group III)	18	16.5 (15–27.1)	18.0 (14.2–28.3)	0.60
	Total	43	15.9 (14.1–27.1)	17.3 (13.6–28.3)	0.72
Exocrine pancreatic	Chronic colonization/infection with *A. xylosoxidans* (Group I)	10	10 (100)	10 (100)	1.00
Insufficiency	Intermittent colonization/infection with *A. xylosoxidans* (Group II)	15	14 (93.3)	14 (93.3)	1.00
	Never colonized/infected with *A. xylosoxidans* (Group III)	18	16 (88.9)	16 (88.9)	1.00
	Total	43	40 (93)	40 (93)	1.00
Number of anual	Chronic colonization/infection with *A. xylosoxidans* (Group I)	9	-	1 (0.33–4)	**0.033***
Hospitalizations	Intermittent colonization/infection with *A. xylosoxidans* (Group II)	15	-	0.66 (0–1.66)	
	Never colonized/infected with *A. xylosoxidans* (Group III)	18	-	0.33 (0–1.33)	
	Total	42	-	0.66 (0–4)	

Results expressed as median and ranges (minimum-maximum) or number (%).
FEV_1: forced expiratory volume in one second at baseline; FVC: forced vital capacity at baseline; BMI: body mass index at baseline.
*Group I # Group III (Dunn's multiple comparison test)

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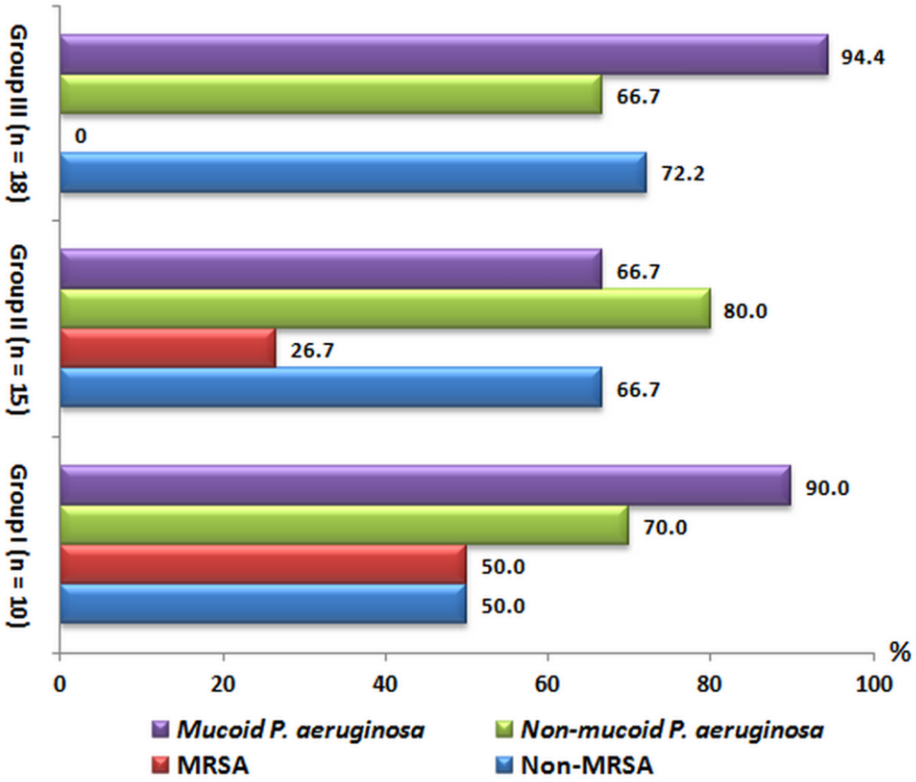
The chronic co-colonization in each group is shown in Figure 1. Regarding chronic co-colonization with methicillin-resistant *S. aureus* (MRSA), a significant difference among the three groups was observed (P=0.002). No significant difference was observed for other types of chronic colonization.

Figure 1. Distribution of chronic co-colonization according to groups: group I, chronic colonization/infection with *A. xylosoxidans* and chronic colonization with *P. aeruginosa*; group II, intermittent colonization/infection with *A. xylosoxidans* and chronic colonization with *P. aeruginosa*; and group III, never colonized/infected with *A. xylosoxidans*, but chronically colonized with *P. aeruginosa*. Regarding the chronic co-colonization with methicillin-resistant *S. aureus* (MRSA), a significant difference was found among the three groups (P=0.002; Group I vs. Group III using Fisher's exact test set for each peer group separately). No significant difference was observed for other types of chronic colonization.



Discussion

The main findings of this study were that in CF patients, a relatively high frequency of *A. xylosoxidans* colonization/infection was present among children, and reduced lung function in patients chronically colonized/infected with *A. xylosoxidans*

was observed. In addition, we did not observe any differences in clinical endpoints over two years when comparing patients chronically colonized with *P. aeruginosa*, with or without *A. xylosoxidans,* except for the increased number of hospital admissions for patients with *A. xylosoxidans*.

In the present study, the frequency of colonization/infection with *A. xylosoxidans* (19.7%) was similar to the upper limit of the range reported in other studies (2% to 17.9%) (7,8). The reported frequency in our study was cumulative, which explains the higher percentages. The large range of *A. xylosoxidans* colonization/infection frequencies may be partly attributed to methodological differences in the studies (6-8,11,12). The prevalence of colonization/infection with *A. xylosoxidans* among pediatric patients (median of seven years of age) was different from that reported in other studies, wherein it was predominantly observed during late adolescence or early adulthood (10,12-14). In this respect, the results most similar to ours are those of a French study that reported a median age of 10.3 years (variation of six to 14 years) for the first positive culture among patients with CF who became chronically colonized/infected with *A. xylosoxidans* (25). However, it is noteworthy that this latest study only includes children and adolescents.

There is no universal criterion for the definition of chronic colonization with *A. xylosoxidans* (6-8,11-13). The criterion of Pereira et al. (24) is more consistent for ensuring chronicity. However, the criterion of chronicity adopted in the present study (21) included an assumption that patient care needs were satisfied; therefore, clinical measures must be adopted during the short period in which colonization can harm the patient. One of the suspected risk factors for colonization/infection with *A. xylosoxidans* is treatment for *P. aeruginosa* (11). Of the 47 patients who had at least one positive culture for *A. xylosoxidans*, 41 were colonized/infected with *P. aeruginosa*, although only 25 met the criteria for chronic colonization/infection with *P. aeruginosa* without BCC colonization. All six patients in whom *P. aeruginosa* was not identified were colonized with BCC. Despite the restriction of the sample size by the selection criteria, chronic colonization with *P. aeruginosa* was considered important for subject pairing because it decreased the chance of bias in the outcomes of interest.

Interestingly, none of the patients in the group chronically colonized/infected with *A. xylosoxidans* were homozygous for the F508del mutation, and its frequency was smaller than that found in the other groups. Therefore, other serious mutations

may be more frequent in this population, as suggested by Cabello et al. (26). With regard to lung function, the FEV_1 values in the group chronically colonized/infected with *A. xylosoxidans* were lower than those found in the other two groups. Although not statistically significant, clinically, this difference suggests a more advanced stage of lung damage among individuals who became chronically colonized/infected with *A. xylosoxidans*. These data are consistent with the hypothesis proposed by De Beats et al. (8), in which individuals with increased lung impairment seem to be more prone to chronic colonization/infection with *A. xylosoxidans*.

A higher frequency of hospitalizations and chronic co-colonization with MRSA were observed in the groups colonized/infected with *A. xylosoxidans* and were more frequent in the chronically colonized/infected group. Zemanick et al. (27) found a higher number of exacerbations requiring intravenous treatment and a higher relative risk of isolation of MRSA, *S. maltophilia*, and *A. xylosoxidans* after the first isolation of *P. aeruginosa*. A recent multicenter study showed that the frequency of colonization with MRSA has increased in recent years and that colonization with *P. aeruginosa* and more intensive therapeutic interventions may be risk factors for chronic colonization with this agent, particularly for healthcare-associated MRSA (HA–MRSA) (28). In our study, although chronic colonization with *P. aeruginosa* was a criterion for pairing, MRSA was not found in the group not colonized/infected with *A. xylosoxidans*.

The present study showed a higher number of hospitalizations in the group chronically colonized/infected with *A. xylosoxidans*. This finding may be explained by the fact that the condition of this group of patients was more severe at the beginning of the study or because other conditions contributed to this outcome. Of note, it was not possible to determine whether the association between chronic colonization with MRSA and *A. xylosoxidans* was the result of increased hospitalization and more intensive antimicrobial therapy or whether any real association existed between these two agents or between these agents and mutations, as previously reported for *P. aeruginosa*.

The group chronically colonized/infected with *A. xylosoxidans* showed much smaller FEV_1 values than the intermittently colonized/infected and non-colonized/infected groups, both at the time of colonization/infection and approximately two years later. Similarly, other authors have found a higher frequency of colonization/infection with *A. xylosoxidans* among individuals with CF with more

severe lung disease (13). Evolutionarily, no significant difference in the intra- or inter-group variation was observed for these parameters. Nevertheless, an analysis of Table 2 indicates that over two years, the FEV_1 values decreased in the group chronically colonized/infected with *A. xylosoxidans* by 6.4% of the predicted value and by 3.8% in the intermittently colonized/infected group. Interestingly, Llorca Otero et al. (29) observed a mean annual decline in FEV_1 of 2.49% in patients chronically colonized/infected with *A. xylosoxidans*.

The strength of the current study is that it is the first Brazilian study that seeks a relationship between clinical data and colonization/infection with *A. xylosoxidans*, which is an emerging pathogen in CF patients investigated worldwide. However, the present study had major limitations. First, the study is limited by the broad age range and small sample size that, at least in part, is explained by its retrospective design and the fact that *A. xylosoxidans* has low incidence/prevalence in CF patients. Second, our population is composed exclusively of patients chronically colonized with *P. aeruginosa*; however, it has been argued that the treatment for chronic colonization with *P. aeruginosa* favors the emergence of *A. xylosoxidans* (10,11). Notwithstanding these limitations, this study can serve as a starting point for future clinical trials that evaluate intervention protocols in CF patients colonized/infected with *A. xylosoxidans*.

In conclusion, a relatively high frequency of *A. xylosoxidans* colonization/infection was present among children, and reduced lung function was observed in patients chronically colonized/infected with *A. xylosoxidans*. This colonized/infected group also showed an increased frequency of chronic colonization with MRSA. In addition, no significant differences in clinical endpoints were observed over two years, except for the increased number of hospitalizations in patients with *A. xylosoxidans*. Regarding the change in lung function over two years, a trend toward a greater decrease in the FEV_1 value of patients chronically colonized/infected with *A. xylosoxidans* was observed.

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5.2 **Artigo 2**

Case reports

Title: *Achromobacter xylosoxidans* infection in cystic fibrosis siblings with different outcomes: case reports

Short running title: *Achromobacter xylosoxidans* in cystic fibrosis

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ABSTRACT

Introduction: The clinical relevance of *Achromobacter xylosoxidans* infection in cystic fibrosis (CF) remains controversial. This emerging agent in CF has been associated with increased lung inflammation, more frequent exacerbations and more severe lung disease. We describe a pair of CF siblings chronically colonized by the same multilocus genotype of *A. xylosoxidans* with different clinical courses, and assess whether this species may have developed any virulence traits and antimicrobial resistance that could have contributed to their singular outcomes.

Case presentation: Two siblings were positive for the F508del and Y1092X mutations, and were chronically colonized by *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The female patient had a more severe CF phenotype and faster clinical deterioration than her brother. Her pulmonary function and computed tomography scan lesions were worse than those of her brother, and both parameters progressively declined. She died at 14 years of age, when he was 18. All isolates of *A. xylosoxidans* were biofilm producers. *Achromobacter xylosoxidans* showed less

swarming motility in the female patient.

Conclusions: Biofilm production and diminution of motility allow persistence. Only swarming motility differed between the isolates recovered from the two siblings, but this finding is not sufficient to explain the different clinical outcomes despite their similar genotypes. Modifier genes, unknown environmental factors and female gender can partially explain differences between these siblings. We were unable to correlate any microbiological findings with their clinical courses, and more translational studies are necessary to decrease the gap of knowledge between laboratory and clinical data to promote better clinical interventions.

Keywords: *Achromobacter* spp.; *Achromobacter xylosoxidans*; Cystic fibrosis

1. Introduction

Achromobacter spp. are emergent pathogens in cystic fibrosis (CF) patients. Among the 18 species described, there are 4 new species: *Achromobacter agilis sp. nov., nom. rev., Achromobacter pestifer sp. nov., nom. rev., Achromobacter kerstersii sp. nov. and Achromobacter deleyi sp. nov.* [1]. The most prevalent species in CF using discriminative molecular tools is *Achromobacter xylosoxidans*, which has been associated with increased lung inflammation [2], more frequent CF exacerbations, and more severe lung disease [3,4]. However, evidence of the clinical relevance of these species remains controversial [5]. *Achromobacter ruhlandii, Achromobacter insuavis, Achromobacter dolens* and a few of other *Achromobacter* species may also chronically colonize CF patients, but most are sporadic [6]. The adaptation of *Achromobacter* species to the human host in chronic infection remains

uncharacterized, and studies are needed to clarify the pathogenesis of this agent in CF lung disease [7].

We describe 2 cases of CF siblings chronically colonized with *A. xylosoxidans* for more than 10 years who had different clinical courses and assess whether this species may have developed any coping virulence traits and antimicrobial resistance that could have contributed to their singular outcomes. To measure differences in their clinical courses, we reviewed clinical data, computed tomography (CT) scans and lung function. To investigate the possible role of *A. xylosoxidans* in the etiopathology of lung disease, we investigated the presence of well-known virulence traits favouring bacterial colonization of the host mucosa, such as biofilm formation, bacterial motility and antibiotic resistance.

2. Case reports

Two siblings were diagnosed with CF in the same year, when the younger, a female patient, was 15 months old and her brother was 5 years and 10 months old. Both patients always lived with their parents in the same home where they were born. Both were positive for the F508del and Y1092X severe mutations, had exogenous pancreatic insufficiency and elevated sweat chloride concentrations and were chronically colonized by *A. xylosoxidans*, *Pseudomonas aeruginosa* (both mucoid and non-mucoid simultaneously from the first colonization) and methicillin-susceptible *Staphylococcus aureus* for more than 10 years [8]. However, they had different clinical courses.

Follow-up at the CF reference centre began when the girl was 15 months old, immediately after diagnosis. She had a past history of meconium ileus (MI), gastroesophageal reflux, failure to thrive, one hospitalization for oedema,

hypoproteinaemia and anaemia and an episode of distal intestinal obstruction syndrome. Her parents also related daily productive cough in the previous 2 months, recurrent vomitus and 4 evacuations/day, with greasy malodorous stools. On physical examination, she weighed 6950 g, with a length of 72 cm and body mass index (BMI) of 13.4 kg/m^2 (all less than the 3rd percentile for age and z score -2.01, -2.73 and -2.11 respectively), 44 breaths per minute, subcostal retractions, pulmonary rhonchi, 98% oxygen saturation and hepatomegaly. Oropharyngeal swab culture was positive for *P. aeruginosa* and *S. aureus.* Attempts to eradicate *P. aeruginosa* failed. After 1 year of *P. aeruginosa* and *S. aureus* chronic colonization, at 2 years and 3 months of age, she had the first positive culture for *A. xylosoxidans,* which also evolved to chronic infection. Regarding sporadic colonization, the girl had a single positive sputum culture for *Haemophilus* and one for *Acinetobacter*. In the first 2 years of follow-up in the CF care centre, she was hospitalized 2 times yearly because of CF exacerbation. The period between 3–10 years of age was almost free of bad events, except for persistent difficulty in weight gain, with one hospitalization because of CF exacerbation. Thereafter, her clinical condition steadily worsened. In the next 4 years, she was hospitalized 8 times for pulmonary exacerbations, of which 5 occurred during the last year, with progressive deterioration of lung disease and evolution to respiratory failure. She was referred to a lung transplant centre, but died before transplant at 14 years and 4 months of age.

Her brother, 4 years and 7 months older, was diagnosed with CF after her diagnosis. He was born at term without complications and had a past history of frequent vomiting and poor weight gain since his first months of life. He was hospitalized for dehydration at 3 months of age and for enterorrhagia by Meckel's diverticulum at 6 months of age. At 2 years of age, recurrent upper respiratory tract

infections started. Despite a voracious appetite, his difficulty in weight gain worsened. In the first consultation at the CF reference centre, at 5 years and 10 months of age, his parents reported that he had frequent coughing. On physical examination, he exhibited pallor, with a weight of 15.7 kg (2nd percentile for age), height of 109 cm (11th percentile for age), BMI of 13.3 kg/m^2 (3rd percentile for age), and z scores of -1.22, -1.94 and -1.81, respectively. From the first consultation, *P. aeruginosa* and *S. aureus* respiratory chronic colonization were detected and, from the following month, also *A. xylosoxidans.* Attempts to eradicate *P. aeruginosa* also failed. Regarding sporadic colonization, the boy had two positive cultures for *Haemophilus*. Until 18 years of age, he had 3 exacerbations of CF treated with hospitalization for 14 days each during the 7th and 8th years and another hospitalization for viral encephalitis at age 11. In the last year, at 17 years and 6 months old, liver disease was detected, and at 18 years of age he was admitted twice: once for vasculitis and another for exacerbation of CF. However, their mother claimed that she could not understand the greater debilitation of her daughter's health compared with her son's, despite the daughter's better compliance with the treatment regimen.

The comparisons of lung function (forced expiratory volume in one second - FEV_1), BMI and Bhalla CT score in two siblings were done at the same age and are presented in Fig. 1. Although both siblings exhibited some improvement in their absolute BMI during follow-up at the reference centre, the female's values were significantly lower than the male's (Mann-Whitney test, $p < 0.0001$; interquartile range (IQR) - male: 2.5 kg/m^2, female: 1.1 kg/m^2).

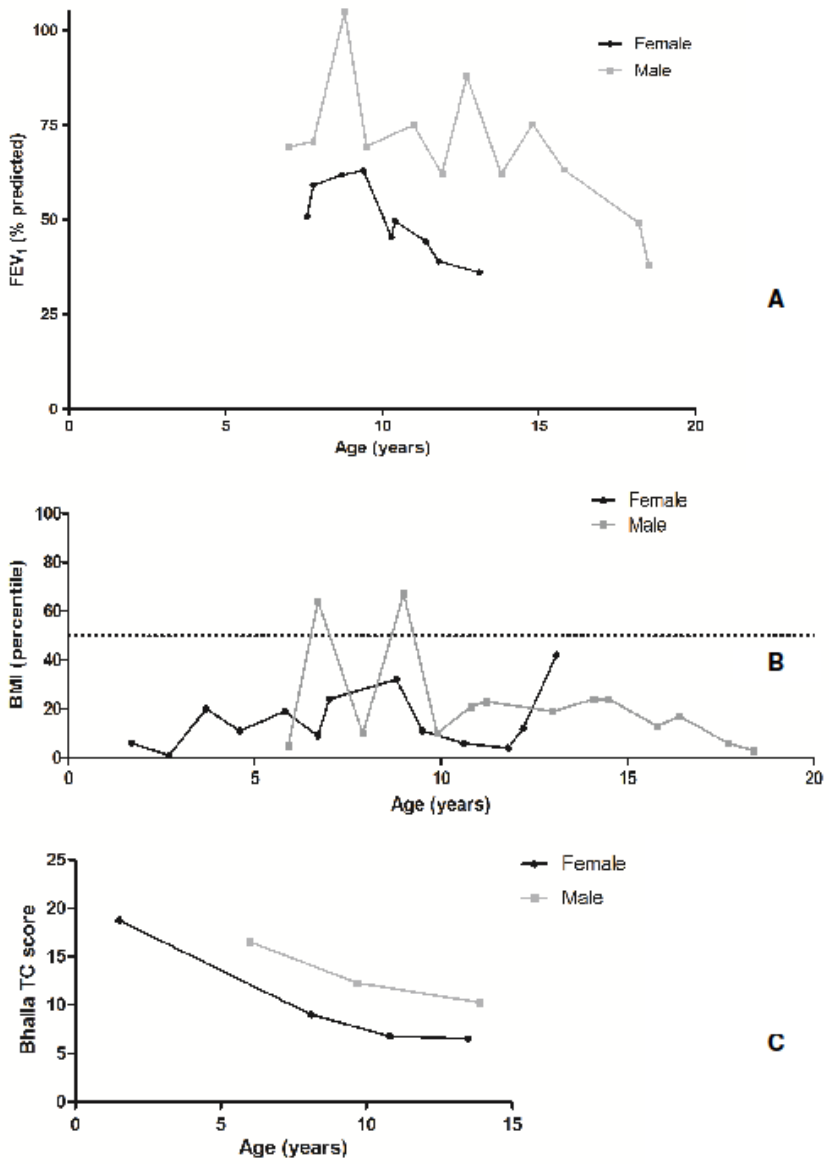

Fig. 1. Evolution of forced expiratory volume in one second (FEV_1 % predicted) **(A)**, body mass index (BMI) **(B)** and Bhalla computed tomography score **(C)** in two siblings with cystic fibrosis. The target of 50th percentile of BMI for age is showed on the dotted line **(B)**. She never achieved the target of 50th percentile of BMI for age, which he passed eventually in the first 5 years of follow-up. The female's FEV_1 was

50.9% at her first spirometry at 7 years and 7 months of age. Her FEV_1 improved to 62.9% in 2 years and declined to 36% in the next 42 months. At 7 years of age, his FEV_1 was 69.1%, and in the next 2 years, it improved to the normal range. However, between 15 and 18 years of age, there was a faster FEV_1 decline to 38%. During the 6 years we were able to measure the lung function of both siblings, his FEV_1 measures were 35% higher than hers. However, the female also exhibited a faster decline of FEV_1 in the last 4 years, to 32%.

To measure lung structural damage, 2 radiologists who were blinded to any other information reviewed the CT scans separately. They assigned modified Bhalla scores [9,10] for each CT scan and reassigned the scores 1 month later. The median of the final Bhalla scores was obtained for each available CT scan. The female had lower Bhalla scores than her brother: 18.7 (1 year and 6 months), 9 (7 year and 2 months), 6.7 (10 years and 11 months) and 6.5 (13 years and 6 months). His scores were 16.5 (5 years and 10 months), 12.2 (9 years and 7 months) and 10.2 (14 years).

Their bacteriological backgrounds were studied between 3.3 and 6.5 years of age for the female and 7.7 and 9.9 years of age for her brother. Species identification was performed by conventional methods, and genotype analysis was performed by multi-locus sequence typing (MLST) of 7 housekeeping genes (*nusA, rpoB, eno, gltB, lepA, nuoL, nrdA*), as previously described [11,12]. Allelic profiles and ST were analysed according to the PubMLST Website (http://pubmlst.org/achromobacter/). All isolates were *A. xylosoxidans* and belonged to the same sequence type (ST 201). This is a new ST previously identified in Brazilian CF patients by our group and added to the PubMLST Website [12].

The determination of minimal inhibitory concentrations (MIC) of antibiotics [ceftazidime (CAZ), ciprofloxacin (CIP), imipenem (IMP) and thimethoprim-sulfamethoxazole (TMP-SXT)] was performed using E-test strips (AB Biodisk, Solna, Sweden). The breakpoints used were those recommended by CLSI for non-*Enterobacteriaceae*. The isolates were considered resistant when MIC ≥ 32 μg/mL for CAZ; MIC ≥ 4 μg/mL for CIP; MIC ≥ 16 μg/mL for IMP and MIC ≥ 4/76 μg/mL for TMP-SXT [13]. The majority of isolates (58.3%) were susceptible to all antibiotics tested (95%CI: 36.5–80.1%). All isolates were susceptible to IMP and CAZ (Fig. 2).

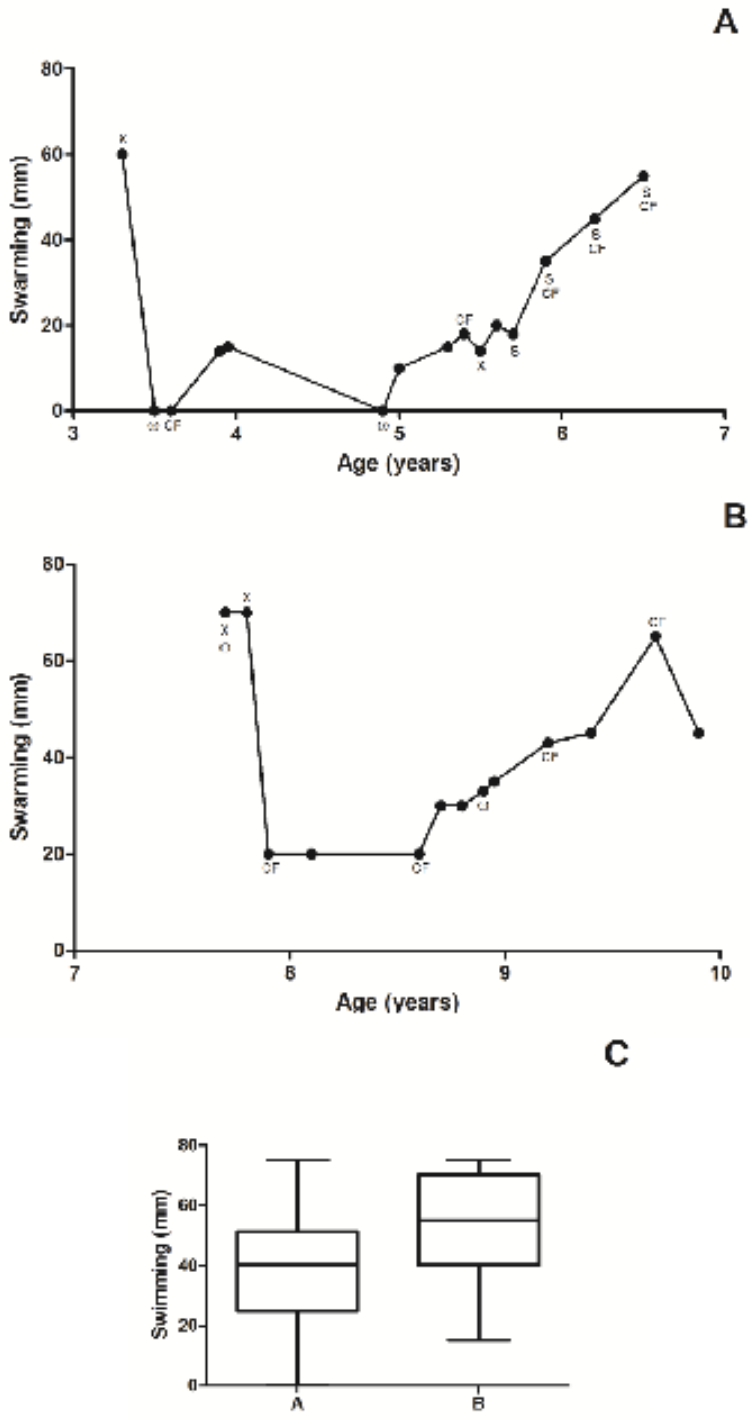
A
Swarming (mm)
Age (years)
B
Swarming (mm)
Age (years)
C
Swimming (mm)
A
B

Fig. 2. Patients' *A. xylosoxidans* characteristics (ST201). Female **(A)**, male **(B)** and box plot diagram showing that swimming did not reach a statistical significant difference **(C)**. Only one colony per sputum of different visits was included for analysis. ω: a weak biofilm producer isolate (all remaining isolates were strong biofilm producers); CF: an isolate resistant to ciprofloxacin; S: an isolate resistant to thimethoprim-sulfamethoxazole (most isolates were susceptible to all antibiotics tested); X: susceptibility data not available.

The swimming and swarming motility abilities were determined as described by Rashid et al. [14]. The results are presented as the median diameters obtained in 3 independent assays performed in triplicate (Fig. 2). There were no differences in swimming phenotype between the isolates (unpaired *t*-test, $p = 0.07$; 95%CI - male: 38.1–58.2 mm, female: 27.2–45.4 mm), but the swarming phenotype was significantly higher for the *A. xylosoxidans* isolates recovered from the male (Mann-Whitney test, $p = 0.004$; IQR - male: 23 mm, female: 20 mm).

Biofilm formation on abiotic plastic surfaces was determined as described by Tendolkar et al. [15]. Microplate wells without bacteria served as negative controls. Then, the *A. xylosoxidans* isolates were divided into different biofilm-producer classes: N, no biofilm producer; W, weak biofilm producer; M, moderate biofilm producer; and S, strong biofilm producer, as described by Stepanovic et al. [16]. All isolates produced biofilms, and the majority were strong producers, but no significant difference in the amount of biofilm formation was detected when isolates from both patients were compared.

Data were analysed with Graph Pad Prism software, version 5.0. The specific tests used are described in the text. Significance was accepted at the $p<0.05$ level.

3. Discussion

The main strength of this study is the attempt to connect microbiological and clinical data to promote the integration of these areas of knowledge and understanding of this complexity. The rapid and deep progress of knowledge are so remarkable that such integration is imperative to reduce doubts and enable effective strategies for clinical interventions. These siblings had the same genetic mutations, lived in the same environment, and were colonized by the same bacterial species, seemingly promising characteristics to "control" possible study bias.

Studies of *A. xylosoxidans* chronic colonization have revealed the persistence of a unique ST revealed by MLST analysis [17,18]. The two siblings shared the same ST, suggesting the possibility of family spread or a common source of acquisition. Transmission in the same family has been described previously by others [17,19,20].

To cause chronic infection, bacteria must overcome the heterogeneous, hostile and stressful lung environment through adaptation mechanisms [21]. Although little is known about the mechanisms of virulence and adaptation of *Achromobacter* spp., studies of *P. aeruginosa* in CF patients have suggested that phenotypes that confer mobility (swimming, swarming and twitching) and toxin production are important for acute infection, whereas biofilm production, the reduction of virulence factors and development of resistance are mechanisms of adaptation that favour chronic infection [14,21]. In our study, more than 50% of isolates from both patients were strong biofilm producers and were susceptible to all antibiotics tested. The significantly lower values of swarming motility of *A. xylosoxidans* isolated from the female patient suggests a loss of motility as a bacterial strategy to reduce virulence and to avoid host immune attack in favour of chronicity [22]. Similar adaptive mechanisms have been observed in studies focused on *P. aeruginosa* and other species [14,21,22]. It is

interesting to note the variability observed between successive isolates for some of the characteristics studied, such as changes in drug resistance and swarming. Since only one colony per specimen was studied, intra-specimen diversity could probably be one hypothesis to explain these observations.

A few translational studies have attempted to connect microbiological and clinical data. Trancassini et al. [7] correlated strong *A. xylosoxidans* biofilm producers with severe obstruction in lung function. Although the present study design did not permit the inference of a cause-and-effect relationship, it supports the chronicity of *A. xylosoxidans* colonization and subsequent deterioration of pulmonary function and structural pulmonary damage. Pulmonary function worsened to severe obstruction in both patients, and both also gradually lost points in the Bhalla CT score.

Various CT scores can be used to assess the presence, severity and extent of pulmonary structural damage and have been correlated with lung function [22]. The Bhalla score is one of the most studied. We used only the final (total) Bhalla score based on its higher levels of reproducibility compared with each component of the score [23]. In addition, the available CT scans were acquired using different protocols, which could favour intra- and interobserver bias for fine details, thus further supporting the use of only the final score.

The combination of the F508del and Y1092X genetic mutations is expected to cause pancreatic insufficiency, as observed in these siblings [24]. However, the CF phenotype of the female patient was more severe since birth, and she had a markedly worse outcome than her brother. Siblings with CF often have different phenotypes and clinical courses, even in the presence of the same disease-causing genes, because the CF phenotype is affected not only by the CFTR genotype but also by environmental and other genetic factors [25]. MI, for example, occurs in almost 20%

of CF patients and is more frequent with some CFTR mutations (F508del, G542X, W1282X, R553X, and G551D) but does not necessarily affect multiple siblings. This phenotypic variability has been partially attributed to modifier genes [26,27]. The female gender may also have influenced the outcome as a negative prognostic factor. Despite an equal prevalence of CF, females have a lower median life expectancy than males and a higher risk of respiratory infections [28]. Females acquire *P. aeruginosa* earlier and exhibit a more rapid decline of lung function once infected [29]. The mechanisms underlying this gender disparity has not been fully elucidated, but sex hormones play a role. Estrogen enhances the conversion of *P. aeruginosa* from the non-mucoid to mucoid phenotype, which is more resistant to antibiotics [30]. Studies also suggest that women have more frequent CF exacerbations during ovulation, a period of high estrogen levels. Harness-Bronley et al. [29] analysed a large cohort of patients from the United States Cystic Fibrosis Foundation Patient Registry and found that not only *P. aeruginosa* but also methicillin-susceptible *S. aureus*, methicillin-resistant *S. aureus*, *Haemophilus influenzae*, *A. xylosoxidans*, *Burkholderia cepacia*, *Aspergillus* species, and non-tuberculous mycobacterium are acquired by females at earlier ages, often even before puberty.

Like any study, ours has limitations. First, there was no overlap of periods of microbiological follow-up for the two siblings. Only a period of microbiological data was available that were obtained prior to objective clinical data, such as data related to CT scans and pulmonary function, thus limiting the potential for translational conclusions. Second, we were unable to determine the precise timing of the initial *A. xylosoxidans* infection. Third, the bacterial load in both patients would be interesting to complement the comparison between them. In addition, these sibling patients were also chronically colonized by *P. aeruginosa*, which may be a cause of poor outcome.

Thus, more translational studies are needed to better understand the relationships between microbiological and clinical data and potentially improve patient management.

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Conflict of interest

The authors have no potential conflicts of interest to disclose.

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CONCLUSÕES

- Esta tese, com os dois estudos realizados, não evidenciou impacto da infecção por *A. xylosoxidans* no curso clínico de pacientes com FC.
- É possível que a doença pulmonar avançada pela FC seja um fator de risco para infecção por este microrganismo.
- Infecção crônica por *A. xylosoxidans* associou-se com maior número de internações por ano e com coinfecção por MRSA.
- A possibilidade de transmissão interpessoal ou fonte comum de infecção pelo *A. xylosoxidans* foi demonstrada no segundo estudo, uma vez que a bactéria dos dois irmãos pertencia ao mesmo clone.
- Um único clone foi responsável pela infecção crônica.
- Durante a infecção crônica, bactérias semelhantes podem sofrer mecanismos de adaptação singulares em cada hospedeiro.
- Nenhum fator microbiológico foi capaz de justificar o quadro mais grave na paciente do sexo feminino.

CONSIDERAÇÕES FINAIS

A maioria dos estudos sobre infecção crônica por *A. xylosoxidans* não conseguiu evidenciar impacto desta infecção no curso clínico dos indivíduos com FC. No entanto, são estudos quase sempre retrospectivos, com curtos períodos de observação e com populações pequenas. Estudos de incidência para germes emergentes de baixa frequência, como *A. xylosoxidans*, em doença rara e com população em constante mudança, como a fibrose cística, são dispendiosos, complexos e demandam muito tempo. No entanto, já há evidências de aumento da inflamação pulmonar após a infecção por *A. xylosoxidans* na FC e, em um único trabalho um pouco mais duradouro, foi demonstrada queda da função pulmonar mais acelerada. Considerando a fisiopatologia da doença pulmonar na FC estes dados levantam a suspeita de que a falta de evidências possa estar relacionada às metodologias dos estudos. Estudos multicêntricos talvez ajudem a encontrar evidências mais consistentes.

Quanto aos estudos microbiológicos em FC, a evolução de conhecimento é tão dramática com métodos moleculares que muitas dúvidas surgem a cada dia. Para que respostas sejam conseguidas e esta evolução de conhecimento possa trazer benefícios diretos ao cuidado com os pacientes, é urgente a necessidade de estudos translacionais, aproximando médicos e microbiologistas.

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APÊNDICE A – Artigo 1 no formato em que foi publicado

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Clinical impact of *Achromobacter xylosoxidans* colonization/infection in patients with cystic fibrosis

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Abstract

The rate of diagnosis of colonization/infection of the airways with *Achromobacter xylosoxidans* has increased in cystic fibrosis patients, but its clinical significance is still controversial. This retrospective, case-control study aimed to evaluate the clinical impact of *A. xylosoxidans* colonization/infection in cystic fibrosis patients. Individuals who were chronically colonized/infected (n=10), intermittently colonized/infected (n=15), and never colonized/infected with *A. xylosoxidans* (n=18) were retrospectively evaluated during two periods that were 2 years apart. Demographic characteristics, clinical data, lung function, and chronic bacterial co-colonization data were evaluated. Of the total study population, 87% were pediatric patients and 65.1% were female. Individuals chronically colonized/infected with *A. xylosoxidans* had decreased forced expiratory volume in 1 s (51.7% in the chronic colonization/infection group *vs* 82.7% in the intermittent colonization/infection group *vs* 76% in the never colonized/infected group). Compared with the other two groups, the rate of co-colonization with methicillin-resistant *Staphylococcus aureus* was higher in individuals chronically colonized/infected with *A. xylosoxidans* (P=0.002). Changes in lung function over 2 years in the three groups were not significant, although a trend toward a greater decrease in lung function was observed in the chronically colonized/infected group. Compared with the other two groups, there was a greater number of annual hospitalizations in patients chronically colonized/infected with *A. xylosoxidans* (P=0.033). In cystic fibrosis patients, there was an increased frequency of *A. xylosoxidans* colonization/infection in children, and lung function was reduced in patients who were chronically colonized/infected with *A. xylosoxidans*. Additionally, there were no differences in clinical outcomes during the 2-year period, except for an increased number of hospitalizations in patients with *A. xylosoxidans*.

Key words: Cystic fibrosis; *Achromobacter* spp.; *Achromobacter xylosoxidans*; Microbiology

Introduction

The genus *Achromobacter* contains genetically distinct species and subspecies, and has not been fully characterized (1–5). *Achromobacter* spp. are Gram-negative, aerobic, nonfermenters of glucose bacilli that are widely distributed in the environment. *Achromobacter xylosoxidans* is the most common bacillus in clinical samples and is recognized as an emerging and multidrug-resistant microorganism that causes various opportunistic infections and nosocomial outbreaks (3,6). Most knowledge on *A. xylosoxidans* has been obtained from studies on populations living in regions where cystic fibrosis (CF) is prevalent (3,6).

The rate of colonization/infection with *A. xylosoxidans* in individuals with CF varies between 2% and 17.9% (7,8) and is increasing worldwide. However, this frequency may be underestimated because this organism can be confused with *Pseudomonas aeruginosa*, bacteria from the *Burkholderia cepacia* complex (BCC), and *Stenotrophomonas maltophilia*, particularly in laboratories that are not specialized for evaluation of CF (9).

The factors that predispose patients to colonization/infection have not been fully determined. Frequent exposure to antibiotics, particularly during treatment for chronic colonization with *P. aeruginosa*, may favor the emergence of this and other Gram-negative, multidrug-resistant bacteria (10,11). The possibility of person-to-person transmission, the association of *A. xylosoxidans* colonization/infection with pulmonary inflammation, and an increased frequency of exacerbations have been demonstrated. However, the clinical impact of colonization/infection of *A. xylosoxidans* in CF patients is still controversial (6,11–15). Therefore, the present study aimed to evaluate the clinical impact of *A. xylosoxidans* colonization/infection in patients with CF.

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Material and Methods

Study design

This retrospective, case-control study evaluated patients with a confirmed diagnosis of CF (16). These patients were regularly monitored at the Instituto Fernandes Figueira, Fundação Oswaldo Cruz and Policlínica Piquet Carneiro, Universidade do Estado do Rio de Janeiro (Brazil). Patients' respiratory secretion culture results were obtained between January 2003 and December 2011 at the Laboratório de Bacteriologia, Hospital Universitário Pedro Ernesto (LBACT-UERJ).

The protocol conformed to the World Medical Association Declaration of Helsinki and was approved by the Research Ethics Committee of the Universidade do Estado do Rio de Janeiro (No. CAAE: 00716512.0.3001.5269).

Patients

A total of 238 individuals (155 females and 83 males) with CF were regularly monitored in these referral centers, of whom 25% were adults ($\geqslant$18 years). The routine follow-up period consisted of quarterly consultations, except for infants, who were monitored monthly. The interval between consultations was shortened depending on clinical need. At each visit, the general medical condition, weight, height, and lung function of patients were evaluated; and respiratory secretions were obtained for culture (sputum or oropharyngeal swab for non-expectorating children). All material obtained at these centers was sent to the LBACT-UERJ. In this institution, cultures of respiratory secretions were carried out according to standardized protocols established for CF patients. Cultures were performed every 3 months throughout the study (17).

Identification of *Achromobacter*

Phenotypic methods. Isolates that were identified as *Achromobacter* spp. by the Vitek 2 Compact system using Gram-negative cards (reference no. 21341; bioMérieux, France) were subjected to further identification via a large panel of phenotypic tests, as previously described (18,19).

Molecular methods. To identify each isolate, DNA was extracted by the boiling lysis method, and the entire *16S* rRNA gene was amplified by PCR, sequenced, and used for BLAST searches against the GenBank database (20). The presence of the *A. xylosoxidans* species-specific marker *blaOXA-114* was investigated by PCR amplification as described by Barrado et al. (6). After amplification, the PCR products were sequenced and compared with sequences in the GenBank database at the NCBI using BLAST.

Inclusion and exclusion criteria

The respiratory secretion culture results of patients with CF were evaluated using the LBACT-UERJ database. The inclusion criteria were as follows: 1) patients with one or more cultures that were positive for *A. xylosoxidans* (the term "colonization/infection" is used in reference to positive cultures), and 2) patients who were colonized/infected with *A. xylosoxidans* and chronically colonized with *P. aeruginosa*, defined as more than 50% of cultures positive for the latter agent during 1 year (21). The exclusion criteria consisted of colonization with BCC bacteria and/or the absence of chronic colonization with *P. aeruginosa*.

Definition of the groups

Patients were subdivided according to their *A. xylosoxidans* colonization/infection status into a chronically colonized/infected group and an intermittently colonized/infected group. The criterion for chronic colonization/infection by *A. xylosoxidans* was the same as that adopted for *P. aeruginosa* (21). Any shorter frequency was considered to be intermittent colonization/infection. The control group consisted of individuals who never had a positive culture for *A. xylosoxidans,* and subjects were matched with those in the case groups according to age ($\pm$1 year), sex, and chronic colonization with *P. aeruginosa*. All of the patients were chronically colonized by *P. aeruginosa*, and the status of *A. xylosoxidans* (chronic, intermittent, and never) was defined as described previously. Therefore, the three study groups were as follows: group I, chronic colonization/infection with *A. xylosoxidans* and chronic colonization with *P. aeruginosa*; group II, intermittent colonization/infection with *A. xylosoxidans* and chronic colonization with *P. aeruginosa*; and group III, never colonized/infected with *A. xylosoxidans,* but chronically colonized by *P. aeruginosa*.

Clinical outcomes

The general population was described according to the demographic characteristics, diagnostic criteria for CF, and the presence of exocrine pancreatic insufficiency, cystic fibrosis-related diabetes, and liver disease. The frequency of the *F508del* mutation was described when available. In addition, other chronic bacterial co-colonizations were recorded by adopting the same criteria for chronic colonization as those used for *P. aeruginosa* (21).

Cross-sectional registration of clinical data was performed on two occasions: when the first positive culture for *A. xylosoxidans* occurred (moment 1 [M1]) and as close as possible to 24 months after the first positive culture (moment 2 [M2]). In the control group, data from M1 were paired with those of subjects in the case groups (groups II and III), and the same criteria were followed for M2.

With regard to lung function, the values of forced expiratory volume in 1 s (FEV_1) and forced vital capacity (FVC) were recorded for all patients who were old enough to perform these tests. We recorded the best lung function value that was closest to the time of initial colonization. Similarly, we also recorded the best lung function that was obtained closest to 24 months later. The measurements were obtained with the HD CPL model (nSpire Health, Inc., USA) following the appropriate standards set by the American Thoracic Society (22). The pulmonary function

results are reported as a percentage of the predicted values for the Brazilian population (23). The weight and height of patients were used to calculate body mass index (BMI). FEV_1, FVC, and BMI were compared for the two time periods within and between groups. The median number of annual admissions was also compared between the groups.

Statistical analysis

Numerical data are reported as means ± SD or medians and ranges (minimum–maximum). Categorical data are reported as frequencies (%). The variables had a non-normal distribution according to the Kolmogorov-Smirnov test. Therefore, a non-parametric test was applied. Kruskal-Wallis ANOVA, with the corresponding Dunn's multiple comparison test, was used to compare numerical variables between the three groups. Fisher's exact test was used to compare categorical variables. When the association between categorical variables within the group was significant at 5%, Fisher's exact test, set for each peer group separately, was used. Therefore, we aimed to identify which groups differed from each other at a level of 1.7%. A level of 1.7% (5% divided by the number comparisons: 0.05/3=0.017) was used to control for type I error. To determine the existence of significant variations in FEV_1, FVC, and BMI values between M1 and M2, the Wilcoxon signed rank test was used. Data analysis was performed using SAS software version 6.11 (SAS Institute, Inc., USA). The level of statistical significance was set at $P < 0.05$.

Results

Of the 238 individuals with culture results, 47 (19.7%) had at least one positive culture for *A. xylosoxidans*, among whom 25 met the inclusion criteria for the study. Ten patients were classified as chronically colonized/infected and 15 were classified as intermittently colonized/infected. The control group consisted of 18 patients. No participants died during the study period. The general characteristics of the study population and comparison between groups at baseline are reported in Table 1.

The median period of chronic colonization with *P. aeruginosa* was 1 year, and this ranged from 1 to 3 years. The baseline values for age, sex, *F508del* mutation frequency, exocrine pancreatic insufficiency, diabetes, liver disease, length of colonization with *P. aeruginosa*, and BMI were similar among the three groups. FEV_1 and FVC values were lower in the chronically colonized/infected group, but this difference was not significant compared with the other groups (Table 1).

When the two periods (M1 and M2) were compared, there was a significant increase in FEV_1 (P=0.014) and significant reduction in FVC (P=0.016) for the total sample. However, no significant changes were observed for these parameters for the patient groups. The median number of annual admissions during the study period was significantly different between the groups (P=0.033). There was a higher number of annual admissions in the chronically colonized/infected group compared to the never colonized/infected group. Information regarding

Table 1. General characteristics of the study population and comparison between groups at baseline.

Variables	Total sample		Group I		Group II		Group III		P
	n		n		n		n		
Age (years)	43	7 (1–37)	10	10.5 (3–18)	15	7 (2–33)	18	7.5 (1–37)	0.64
Gender (female)	43	28 (65.1)	10	5 (50)	15	9 (60)	18	14 (77.8)	0.29
Family history	43	4 (9.3)	10	2 (20)	15	1 (6.7)	18	1 (5.6)	0.43
Neonatal screening	43	5 (11.6)	10	1 (10)	15	1 (6.7)	18	3 (16.7)	0.83
Homozygous *F508del* frequency	37	9 (24.3)	8	0 (0)	13	5 (38.5)	16	4 (25)	0.14
Heterozygous *F508del* frequency	37	16 (43.2)	8	3 (37.5)	13	7 (53.9)	16	6 (37.5)	0.69
Another mutation/mutation unidentified	37	12 (32.4)	8	5 (62.5)	13	1 (7.7)	16	6 (37.5)	**0.027***
Exocrine pancreatic insufficiency	43	38 (88.4)	10	10 (100)	15	14 (93.3)	18	14 (77.8)	0.26
Cystic fibrosis-related diabetes	43	2 (4.6)	10	1 (10)	15	1 (6.7)	18	0 (0)	0.72
Liver disease	43	1 (2.3)	10	0 (0)	15	1 (6.7)	18	0 (0)	0.78
Chronic *P. aeruginosa* time (years)	43	1 (1–3)	10	1.5 (1–3)	15	1 (1–2)	18	1 (1–3)	0.23
FEV_1-M1	24	70.1 (27.3–112)	6	51.7 (27.3–95.1)	6	82.7 (55.1–112)	12	76 (35–108.5)	0.15
FVC-M1	24	86.4 (41.9–115)	6	67.8 (41.9–90.7)	6	98.7 (76.1–110)	12	82.7 (49.9–115)	0.09
BMI-M1	43	15.9 (11.5–27.1)	10	15.4 (14.1–23.4)	15	16.2 (13.6–22.9)	18	16.4 (11.5–27.1)	0.31

Results are reported as median and ranges (minimum–maximum) or number (%). Group I: chronic colonization/infection with *A. xylosoxidans*; group II: intermittent colonization/infection with *A. xylosoxidans*; group III: never colonized/infected with *A. xylosoxidans*. FEV_1-M1: forced expiratory volume in 1 s at baseline; FVC-M1: forced vital capacity at baseline; BMI-M1: body mass index at baseline. *Significant difference was observed between groups I and II (Fisher's exact test set for each peer group separately).

pulmonary function, BMI, and clinical data for each group at M1 and M2 is reported in Table 2.

Chronic co-colonization in each group is shown in Figure 1. A significant difference (P=0.002) in chronic co-colonization with methicillin-resistant *S. aureus* (MRSA) among the three groups was observed. Chronic co-colonization with MRSA was observed in 50% and 26.7% of the patients who were chronically colonized/infected with *A. xylosoxidans* and intermittently colonized/infected with *A. xylosoxidans*, respectively. No patients without colonization/infection with *A. xylosoxidans* had chronic co-colonization with MRSA. No significant difference was observed for other types of chronic colonization.

Discussion

The main findings of this study were as follows: in CF patients, a relatively high frequency of *A. xylosoxidans* colonization/infection was present among children, and reduced lung function in patients who were chronically colonized/infected with *A. xylosoxidans* was observed. In addition, we did not observe any differences in clinical endpoints over 2 years when we compared patients who were chronically colonized with *P. aeruginosa*, with or without *A. xylosoxidans,* except for an increased number of hospital admissions for patients with *A. xylosoxidans*.

In the present study, the frequency of colonization/infection with *A. xylosoxidans* (19.7%) was similar to the upper limit of the range reported in other studies (2% to 17.9%) (7,8). This reported frequency in our study was cumulative, which explains the higher percentage than other studies. The large range in *A. xylosoxidans* colonization/infection frequency may be partly attributed to methodological differences between the studies (6–8,11,12). The prevalence of colonization/infection with *A. xylosoxidans* in pediatric patients in our study (median, 7 years) was different from that reported in other studies, which showed that it was predominantly observed during late adolescence or early adulthood (10,12–14). The most similar results to our study are those of a French study that reported a median age of 10.3 years (variation of 6 to 14 years) for the first positive culture among patients with CF who became chronically colonized/infected with *A. xylosoxidans* (24). However, notably, this French study only included children and adolescents.

Table 2. Lung function, body mass index, and clinical data according to the groups at baseline and after 24 months.

Group	n	Baseline	After 24 months	P
FEV_1 (% predicted)				
Chronic colonization/infection with *A. xylosoxidans* (group I)	6	51.7 (27.3–95.1)	45.3 (16.6–88.7)	0.063
Intermittent colonization/infection with *A. xylosoxidans* (group II)	6	82.7 (55.1–112)	78.9 (40.2–125.3)	0.15
Never colonized/infected with *A. xylosoxidans* (group III)	12	76.0 (35–108.5)	82.7 (24–102.7)	0.30
Total	24	70.1 (35–112)	75.5 (16.6–125.3)	**0.014**
FVC (% predicted)				
Chronic colonization/infection with *A. xylosoxidans* (group I)	6	67.8 (55–90.7)	69.5 (27.5–92.5)	0.44
Intermittent colonization/infection with *A. xylosoxidans* (group II)	6	98.7 (76.1–110)	92.1 (61.4–117.5)	0.22
Never colonized/infected with *A. xylosoxidans* (group III)	12	82.7 (35–108.5)	84.1 (25.6–102.5)	0.061
Total	24	86.4 (35–110)	84.4 (25.6–117.5)	**0.016**
BMI (kg/m^2)				
Chronic colonization/infection with *A. xylosoxidans* (group I)	10	15.4 (14.1–23.4)	16.6 (14.1–23.1)	0.94
Intermittent colonization/infection with *A. xylosoxidans* (group II)	15	16.2 (14.2–22.9)	16.4 (13.6–22.9)	0.67
Never colonized/infected with *A. xylosoxidans* (group III)	18	16.5 (15–27.1)	18.0 (14.2–28.3)	0.60
Total	43	15.9 (14.1–27.1)	17.3 (13.6–28.3)	0.72
Exocrine pancreatic insufficiency				
Chronic colonization/infection with *A. xylosoxidans* (group I)	10	10 (100)	10 (100)	1.00
Intermittent colonization/infection with *A. xylosoxidans* (group II)	15	14 (93.3)	14 (93.3)	1.00
Never colonized/infected with *A. xylosoxidans* (group III)	18	16 (88.9)	16 (88.9)	1.00
Total	43	40 (93)	40 (93)	1.00
Number of annual hospitalizations				
Chronic colonization/infection with *A. xylosoxidans* (group I)	9	–	1 (0.33–4)	**0.033***
Intermittent colonization/infection with *A. xylosoxidans* (group II)	15	–	0.66 (0–1.66)	
Never colonized/infected with *A. xylosoxidans* (group III)	18	–	0.33 (0–1.33)	
Total	42	–	0.66 (0–4)	

Results are reported as median and ranges (minimum–maximum) or number (%). FEV_1: forced expiratory volume in 1 s at baseline; FVC: forced vital capacity at baseline; BMI: body mass index at baseline. * Significant difference was observed between groups I and III (Dunn's multiple comparison test).

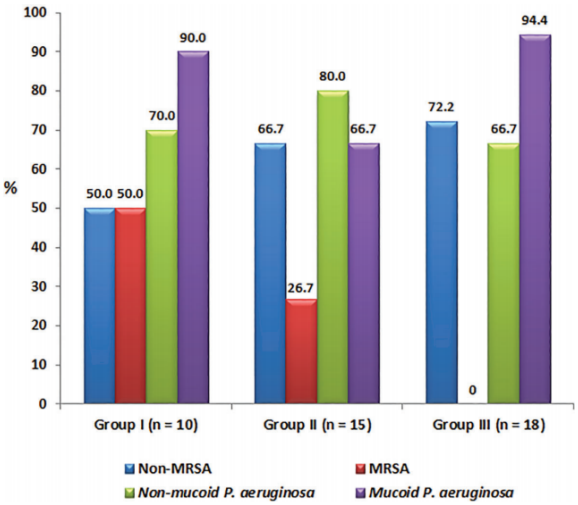

Figure 1. Distribution of chronic co-colonization according to the following groups: group I, chronic colonization/infection with *A. xylosoxidans* and chronic colonization with *P. aeruginosa*; group II, intermittent colonization/infection with *A. xylosoxidans* and chronic colonization with *P. aeruginosa*; and group III, never colonized/infected with *A. xylosoxidans,* but chronically colonized with *P. aeruginosa*. A significant difference in chronic co-colonization with methicillin-resistant *S. aureus* (MRSA) was found among the three groups (P=0.002; significant difference was observed between groups I and III using Fisher's exact test set for each peer group separately). No significant difference was observed for other types of chronic colonization.

There is no universal criterion for the definition of chronic colonization with *A. xylosoxidans* (6–8,11–13). The criterion of Pereira et al. (25) is more consistent for ensuring chronicity. However, the criterion of chronicity that was adopted in the present study (21) included an assumption that patient care needs were satisfied. Therefore, clinical measures must be adopted during the short period in which colonization can harm the patient. One of the suspected risk factors for colonization/infection with *A. xylosoxidans* is treatment for *P. aeruginosa* (11). In our study, of the 47 patients who had at least one positive culture for *A. xylosoxidans*, 41 were colonized/infected with *P. aeruginosa*. However, only 25 met the criteria for chronic colonization/infection with *P. aeruginosa* without BCC colonization. All six patients in whom *P. aeruginosa* was not identified were colonized with BCC. Despite the restriction of the sample size by the selection criteria, chronic colonization with *P. aeruginosa* was considered important for subject pairing because it decreased the chance of bias in the outcomes of interest.

Interestingly, none of the patients in the group of patients who were chronically colonized/infected with *A. xylosoxidans* were homozygous for the *F508del* mutation, and its frequency was smaller than that found in the other groups. Therefore, other serious mutations may be more frequent in this population, as suggested by Cabello et al. (26). With regard to lung function, FEV_1 values in the group of patients who were chronically colonized/infected with *A. xylosoxidans* were lower than those found in the other two groups. Although this finding was not statistically significant, clinically, this difference suggests a more advanced stage of lung damage among individuals who became chronically colonized/infected with *A. xylosoxidans*. These data are consistent with the hypothesis proposed by De Baets et al. (8), in which individuals with increased lung impairment appear to be more prone to chronic colonization/infection with *A. xylosoxidans*.

A higher frequency of hospitalizations and chronic co-colonization with MRSA was observed in the group of patients who were colonized/infected with *A. xylosoxidans,* and this frequency was highest in the chronically colonized/infected group. Zemanick et al. (27) found a higher number of exacerbations requiring intravenous treatment and a higher relative risk of isolation of MRSA, *S. maltophilia*, and *A. xylosoxidans* after the first isolation of *P. aeruginosa*. A recent multicenter study showed that the frequency of colonization with MRSA has increased in recent years (28). Additionally, colonization with *P. aeruginosa* and more intensive therapeutic interventions may be risk factors for chronic colonization with MRSA, particularly for healthcare-associated MRSA (HA-MRSA) (28). In our study, although chronic colonization with *P. aeruginosa* was a criterion for pairing, MRSA was not found in the group that was not colonized/infected with *A. xylosoxidans*.

Compared with the other two groups, there was higher number of hospitalizations in the chronically colonized/infected group. This finding may be explained by the fact that the condition of this group of patients was more severe at the beginning of the study or because other conditions contributed to this outcome. Notably, we were unable to determine whether the association between chronic colonization with MRSA and *A. xylosoxidans* was the result of

increased hospitalization and more intensive antimicrobial therapy, or whether any real association existed between these two agents or between these agents and mutations, as previously reported for *P. aeruginosa* (29).

The chronically colonized/infected group showed much smaller FEV_1 values than the intermittently colonized/infected and non-colonized/infected groups, at the time of colonization/infection and approximately 2 years later. Similarly, other studies have shown a higher frequency of colonization/infection with *A. xylosoxidans* in individuals with CF with more severe lung disease (13). In the present study, no significant difference in intra- or inter-group variation was observed for these parameters. Nevertheless, over 2 years, FEV_1 values decreased in the chronically colonized/infected group by 6.4% of the predicted value and by 3.8% in the intermittently colonized/infected group (Table 2). Interestingly, Llorca Otero et al. (30) observed a mean annual decline in FEV_1 of 2.49% in patients who were chronically colonized/infected with *A. xylosoxidans*.

A strength of the current study is that it is the first Brazilian study to determine a relationship between clinical data and colonization/infection with *A. xylosoxidans*. However, the present study has major limitations. First, our study was limited by the broad age range and small sample size. These factors can, at least in part, be explained by the study's retrospective design and the fact that *A. xylosoxidans* has a low incidence/prevalence in CF patients. Second, our population was exclusively composed of patients who were chronically colonized with *P. aeruginosa*. However, treatment for chronic colonization with *P. aeruginosa* might favor the emergence of *A. xylosoxidans* (10,11). Notwithstanding these limitations, this study can serve as a starting point for future clinical trials to evaluate intervention protocols in CF patients who are colonized/infected with *A. xylosoxidans*.

In conclusion, a relatively high frequency of *A. xylosoxidans* colonization/infection was present in children; and reduced lung function was observed in patients who were chronically colonized/infected with *A. xylosoxidans*. This colonized/infected group also showed an increased frequency of chronic colonization with MRSA. In addition, no significant differences in clinical endpoints were observed over 2 years, except for an increased number of hospitalizations in patients with *A. xylosoxidans*. With regard to the change in lung function over 2 years, a trend toward a decrease in FEV_1 values of patients who were chronically colonized/infected with *A. xylosoxidans* was observed.

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increased hospitalization and more intensive antimicrobial therapy, or whether any real association existed between these two agents or between these agents and mutations, as previously reported for *P. aeruginosa* (29).

The chronically colonized/infected group showed much smaller FEV_1 values than the intermittently colonized/infected and non-colonized/infected groups, at the time of colonization/infection and approximately 2 years later. Similarly, other studies have shown a higher frequency of colonization/infection with *A. xylosoxidans* in individuals with CF with more severe lung disease (13). In the present study, no significant difference in intra- or inter-group variation was observed for these parameters. Nevertheless, over 2 years, FEV_1 values decreased in the chronically colonized/infected group by 6.4% of the predicted value and by 3.8% in the intermittently colonized/infected group (Table 2). Interestingly, Llorca Otero et al. (30) observed a mean annual decline in FEV_1 of 2.49% in patients who were chronically colonized/infected with *A. xylosoxidans*.

A strength of the current study is that it is the first Brazilian study to determine a relationship between clinical data and colonization/infection with *A. xylosoxidans*. However, the present study has major limitations. First, our study was limited by the broad age range and small sample size. These factors can, at least in part, be explained by the study's retrospective design and the fact that *A. xylosoxidans* has a low incidence/prevalence in CF patients. Second, our population was exclusively composed of patients who were chronically colonized with *P. aeruginosa*. However, treatment for chronic colonization with *P. aeruginosa* might favor the emergence of *A. xylosoxidans* (10,11). Notwithstanding these limitations, this study can serve as a starting point for future clinical trials to evaluate intervention protocols in CF patients who are colonized/infected with *A. xylosoxidans*.

In conclusion, a relatively high frequency of *A. xylosoxidans* colonization/infection was present in children; and reduced lung function was observed in patients who were chronically colonized/infected with *A. xylosoxidans*. This colonized/infected group also showed an increased frequency of chronic colonization with MRSA. In addition, no significant differences in clinical endpoints were observed over 2 years, except for an increased number of hospitalizations in patients with *A. xylosoxidans*. With regard to the change in lung function over 2 years, a trend toward a decrease in FEV_1 values of patients who were chronically colonized/infected with *A. xylosoxidans* was observed.

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Case report

Achromobacter xylosoxidans infection in cystic fibrosis siblings with different outcomes: Case reports

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ABSTRACT

Introduction: The clinical relevance of *Achromobacter xylosoxidans* infection in cystic fibrosis (CF) remains controversial. This emerging agent in CF has been associated with increased lung inflammation, more frequent exacerbations and more severe lung disease. We describe a pair of CF siblings chronically colonized by the same multilocus genotype of *A. xylosoxidans* with different clinical courses, and assess whether this species may have developed any virulence traits and antimicrobial resistance that could have contributed to their singular outcomes.
Case presentation: Two siblings were positive for the F508del and Y1092X mutations, and were chronically colonized by *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The female patient had a more severe CF phenotype and faster clinical deterioration than her brother. Her pulmonary function and computed tomography scan lesions were worse than those of her brother, and both parameters progressively declined. She died at 14 years of age, when he was 18. All isolates of *A. xylosoxidans* were biofilm producers. *Achromobacter xylosoxidans* showed less swarming motility in the female patient.
Conclusions: Biofilm production and diminution of motility allow persistence. Only swarming motility differed between the isolates recovered from the two siblings, but this finding is not sufficient to explain the different clinical outcomes despite their similar genotypes. Modifier genes, unknown environmental factors and female gender can partially explain differences between these siblings. We were unable to correlate any microbiological findings with their clinical courses, and more translational studies are necessary to decrease the gap of knowledge between laboratory and clinical data to promote better clinical interventions.
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1. Introduction

Achromobacter spp. are emergent pathogens in cystic fibrosis (CF) patients. There are 16 species and 2 subspecies already described [1]. Recently there is a proposal to include 4 new species: *Achromobacter agilis* sp. *nov.*, *nom. rev.*, *Achromobacter pestifer* sp. *nov.*, *nom. rev.*, *Achromobacter kerstersii* sp. *nov. and Achromobacter deleyi* sp. *nov.* [2]. The most prevalent species in CF using discriminative molecular tools is *Achromobacter xylosoxidans*, which has been associated with increased lung inflammation [3], more

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frequent CF exacerbations, and more severe lung disease [4,5]. However, evidence of the clinical relevance of these species remains controversial [6]. *Achromobacter ruhlandii, Achromobacter insuavis, Achromobacter dolens* and a few of other *Achromobacter* species may also chronically colonize CF patients, but most are sporadic [7]. The adaptation of *Achromobacter* species to the human host in chronic infection remains uncharacterized, and studies are needed to clarify the pathogenesis of this agent in CF lung disease [8].

We describe 2 cases of CF siblings chronically colonized with *A. xylosoxidans* for more than 10 years who had different clinical courses and assess whether this species may have developed any coping virulence traits and antimicrobial resistance that could have contributed to their singular outcomes. To measure differences in their clinical courses, we reviewed clinical data, computed tomography (CT) scans and lung function. To investigate the possible role of *A. xylosoxidans* in the etiopathology of lung disease, we investigated the presence of well-known virulence traits favouring bacterial colonization of the host mucosa, such as biofilm formation, bacterial motility and antibiotic resistance.

2. Case reports

Two siblings were diagnosed with CF in the same year, when the younger, a female patient, was 15 months old and her brother was 5 years and 10 months old. Both patients always lived with their parents in the same home where they were born. Both were positive for the F508del and Y1092X severe mutations, had exogenous pancreatic insufficiency and elevated sweat chloride concentrations and were chronically colonized by *A. xylosoxidans, Pseudomonas aeruginosa* (both mucoid and non-mucoid from the first colonization) and methicillin-susceptible *Staphylococcus aureus* for more than 10 years [9]. However, they had different clinical courses.

Follow-up at the CF reference centre began when the girl was 15 months old, immediately after diagnosis. She had a past history of meconium ileus (MI), gastroesophageal reflux, failure to thrive, one hospitalization for oedema, hypoproteinaemia and anaemia and an episode of distal intestinal obstruction syndrome. Her parents also related daily productive cough in the previous 2 months, recurrent vomitus and 4 evacuations/day, with greasy malodorous stools. On physical examination, she weighed 6950 g, with a length of 72 cm and body mass index (BMI) of 13.4 kg/m^2 (all less than the 3rd percentile for age and z score −2.01, −2.73 and −2.11 respectively), 44 breaths per minute, subcostal retractions, pulmonary rhonchi, 98% oxygen saturation and hepatomegaly. Oropharyngeal swab culture was positive for *P. aeruginosa* and *S. aureus*. Attempts to eradicate *P. aeruginosa* failed. After 1 year of *P. aeruginosa* and *S. aureus* chronic colonization, at 2 years and 3 months of age, she had the first positive culture for *A. xylosoxidans*, which also evolved to chronic infection. Regarding sporadic colonization, the girl had a single positive sputum culture for *Haemophilus* and one for *Acinetobacter*. In the first 2 years of follow-up in the CF care centre, she was hospitalized 2 times yearly because of CF exacerbation. The period between 3 and 10 years of age was almost free of bad events, except for persistent difficulty in weight gain, with one hospitalization because of CF exacerbation. Thereafter, her clinical condition steadily worsened. In the next 4 years, she was hospitalized 8 times for pulmonary exacerbations, of which 5 occurred during the last year, with progressive deterioration of lung disease and evolution to respiratory failure. She was referred to a lung transplant centre, but died before transplant at 14 years and 4 months of age.

Her brother, 4 years and 7 months older, was diagnosed with CF after her diagnosis. He was born at term without complications and had a past history of frequent vomiting and poor weight gain since his first months of life. He was hospitalized for dehydration at 3 months of age and for enterorrhagia by Meckel's diverticulum at 6 months of age. At 2 years of age, recurrent upper respiratory tract infections started. Despite a voracious appetite, his difficulty in weight gain worsened. In the first consultation at the CF reference centre, at 5 years and 10 months of age, his parents reported that he had frequent coughing. On physical examination, he exhibited pallor, with a weight of 15.7 kg (2nd percentile for age), height of 109 cm (11th percentile for age), BMI of 13.3 kg/m^2 (3rd percentile for age), and z scores of −1.22, −1.94 and −1.81, respectively. From the first consultation, *P. aeruginosa* and *S. aureus* respiratory chronic colonization were detected and, from the following month, also *A. xylosoxidans*. Attempts to eradicate *P. aeruginosa* also failed. Regarding sporadic colonization, the boy had two positive cultures for *Haemophilus*. Until 18 years of age, he had 3 exacerbations of CF treated with hospitalization for 14 days each during the 7th and 8th years and another hospitalization for viral encephalitis at age 11. In the last year, at 17 years and 6 months old, liver disease was detected, and at 18 years of age he was admitted twice: once for vasculitis and another for exacerbation of CF. However, their mother claimed that she could not understand the greater debilitation of her daughter's health compared with her son's, despite the daughter's better compliance with the treatment regimen.

The comparisons of lung function (forced expiratory volume in 1 s - FEV_1), BMI and Bhalla CT score in two siblings were done at the same age and are presented in Fig. 1. Although both siblings exhibited some improvement in their absolute BMI during follow-up at the reference centre, the female's values were significantly lower than the male's (Mann-Whitney test, $p < 0.0001$; interquartile Range (IQR) - male: 2.5 kg/m^2, female: 1.1 kg/m^2).

To measure lung structural damage, 2 radiologists who were blinded to any other information reviewed the CT scans separately. They assigned modified Bhalla scores [10,11] for each CT scan and reassigned the scores 1 month later. The median of the final Bhalla scores was obtained for each available CT scan. The female had lower Bhalla scores than her brother: 18.7 (1 year and 6 months), 9 (7 year and 2 months), 6.7 (10 years and 11 months) and 6.5 (13 years and 6 months). His scores were 16.5 (5 years and 10 months), 12.2 (9 years and 7 months) and 10.2 (14 years).

Their bacteriological backgrounds were studied between 3.3 and 6.5 years of age for the female and 7.7 and 9.9 years of age for her brother. Species identification was performed by conventional methods, and genotype analysis was performed by multi-locus sequence typing (MLST) of 7 housekeeping genes (*nusA, rpoB, eno, gltB, lepA, nuoL, nrdA*), as previously described [12,13]. Allelic profiles and ST were analysed according to the PubMLST Website (http://pubmlst.org/achromobacter/). All isolates were *A. xylosoxidans* and belonged to the same sequence type (ST 201). This is a new ST previously identified in Brazilian CF patients by our group and added to the PubMLST Website [13].

The determination of minimal inhibitory concentrations (MIC) of antibiotics [ceftazidime (CAZ), ciprofloxacin (CIP), imipenem (IMP) and thimethoprim-sulfamethoxazole (TMP-SXT)] was performed using E-test strips (AB Biodisk, Solna, Sweden). The breakpoints used were those recommended by CLSI for non-*Enterobacteriaceae*. The isolates were considered resistant when MIC ≥32 μg/mL for CAZ; MIC ≥4 μg/mL for CIP; MIC ≥16 μg/mL for IMP and MIC ≥4/76 μg/mL for TMP-SXT [14]. The majority of isolates (58.3%) were susceptible to all antibiotics tested (95%CI: 36.5–80.1%). All isolates were susceptible to IMP and CAZ (Fig. 2).

The swimming and swarming motility abilities were determined as described by Rashid et al. [15]. The results are presented as the median diameters obtained in 3 independent assays performed in triplicate (Fig. 2). There were no differences in swimming phenotype between the isolates (unpaired *t*-test, $p = 0.07$; 95%CI - male: 38.1–58.2 mm, female: 27.2–45.4 mm), but the swarming

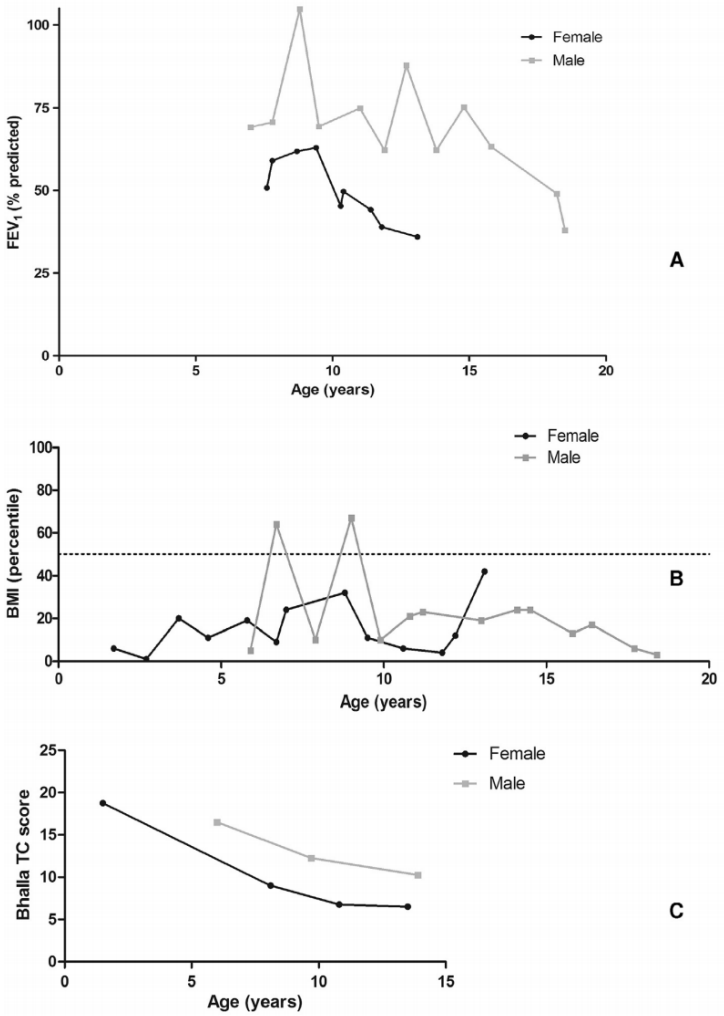

Fig. 1. Evolution of forced expiratory volume in 1 s (FEV_1% predicted) **(A)**, body mass index (BMI) **(B)** and Bhalla computed tomography score **(C)** in two siblings with cystic fibrosis. The target of 50th percentile of BMI for age is showed on the dotted line **(B)**. She never achieved the target of 50th percentile of BMI for age, which he passed eventually in the first 5 years of follow-up. The female's FEV_1 was 50.9% at her first spirometry at 7 years and 7 months of age. Her FEV_1 improved to 62.9% in 2 years and declined to 36% in the next 42 months. At 7 years of age, his FEV_1 was 69.1%, and in the next 2 years, it improved to the normal range. However, between 15 and 18 years of age, there was a faster FEV_1 decline to 38%. During the 6 years we were able to measure the lung function of both siblings, his FEV_1 measures were 35% higher than hers. However, the female also exhibited a faster decline of FEV_1 in the last 4 years, to 32%.

phenotype was significantly higher for the *A. xylosoxidans* isolates recovered from the male (Mann-Whitney test, $p = 0.004$; IQR - male: 23 mm, female: 20 mm).

Biofilm formation on abiotic plastic surfaces was determined as described by Tendolkar et al. [16]. Then, the *A. xylosoxidans* isolates were divided into different biofilm-producer classes: N, no biofilm producer; W, weak biofilm producer; M, moderate biofilm producer; and S, strong biofilm producer, as described by Stepanovic et al. [17]. All isolates produced biofilms, and the majority were strong producers, but no significant difference in the amount of biofilm formation was detected when isolates from both patients were compared.

Data were analysed with Graph Pad Prism software, version 5.0. The specific tests used are described in the text. Significance was accepted at the $p < 0.05$ level.

3. Discussion

The main strength of this study is the attempt to connect microbiological and clinical data to promote the integration of these areas of knowledge and understanding of this complexity. The rapid and deep progress of knowledge are so remarkable that

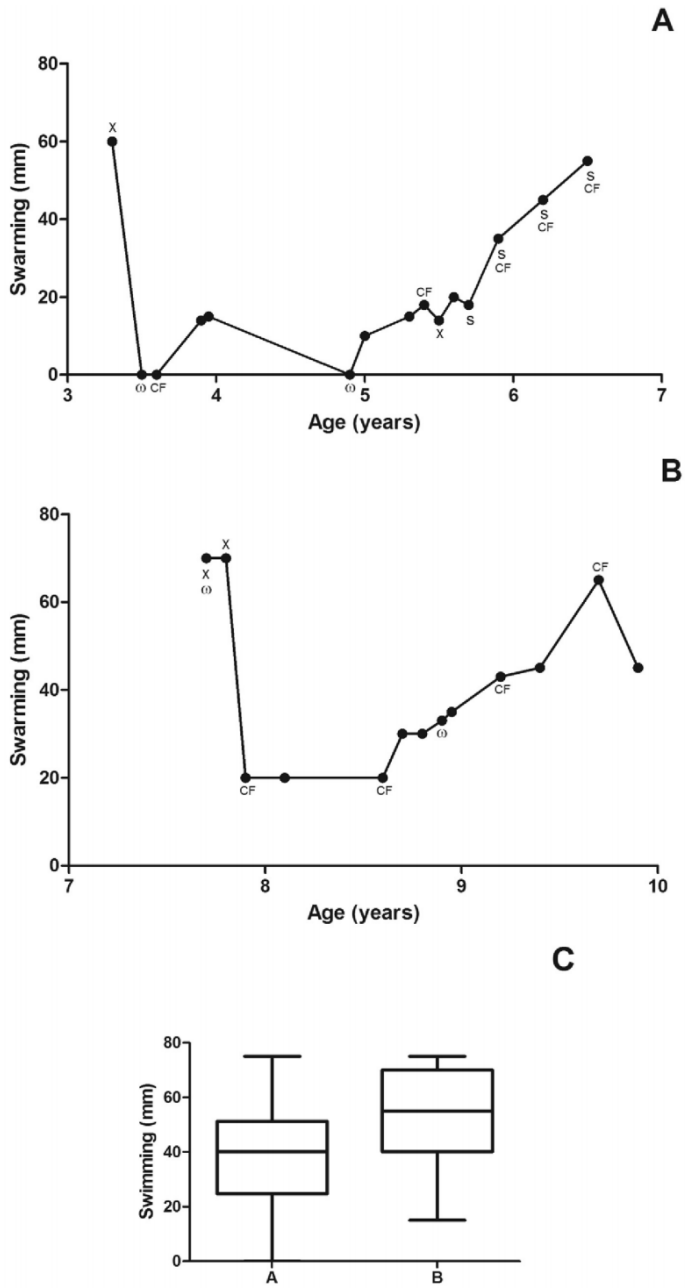

Fig. 2. Patients' *A. xylosoxidans* characteristics (ST201). Female **(A)**, male **(B)** and box plot diagram showing that swimming did not reach a statistical significant difference **(C)**. Only one colony per sputum of different visits was included for analysis. ω: a weak biofilm producer isolate (all remaining isolates were strong biofilm producers); CF: an isolate resistant to ciprofloxacin; S: an isolate resistant to thimethoprim-sulfamethoxazole (most isolates were susceptible to all antibiotics tested); X: susceptibility data not available.

such integration is imperative to reduce doubts and enable effective strategies for clinical interventions. These siblings had the same genetic mutations, lived in the same environment, and were colonized by the same bacterial species, seemingly promising characteristics to "control" possible study bias.

Studies of *A. xylosoxidans* chronic colonization have revealed the persistence of a unique ST revealed by MLST analysis [18,19]. The two siblings shared the same ST, suggesting the possibility of family spread or a common source of acquisition. Transmission in the same family has been described previously by others [18,20,21].

To cause chronic infection, bacteria must overcome the heterogeneous, hostile and stressful lung environment through adaptation mechanisms [22]. Although little is known about the mechanisms of virulence and adaptation of *Achromobacter* spp., studies of *P. aeruginosa* in CF patients have suggested that phenotypes that confer mobility (swimming, swarming and twitching) and toxin production are important for acute infection, whereas biofilm production, the reduction of virulence factors and development of resistance are mechanisms of adaptation that favour chronic infection [15,22]. In our study, more than 50% of isolates from both patients were strong biofilm producers and were susceptible to all antibiotics tested. The significantly lower values of swarming motility of *A. xylosoxidans* isolated from the female patient suggests a loss of motility as a bacterial strategy to reduce virulence and to avoid host immune attack in favour of chronicity [23]. Similar adaptive mechanisms have been observed in studies focused on *P. aeruginosa* and other species [15,22,23]. It is interesting to note the variability observed between successive isolates for some of the characteristics studied, such as changes in drug resistance and swarming. Since only one colony per specimen was studied, intra-specimen diversity could probably be one hypothesis to explain these observations.

A few translational studies have attempted to connect microbiological and clinical data. Trancassini et al. [8] correlated strong *A. xylosoxidans* biofilm producers with severe obstruction in lung function. Although the present study design did not permit the inference of a cause-and-effect relationship, it supports the chronicity of *A. xylosoxidans* colonization and subsequent deterioration of pulmonary function and structural pulmonary damage. Pulmonary function worsened to severe obstruction in both patients, and both also gradually lost points in the Bhalla CT score.

Various CT scores can be used to assess the presence, severity and extent of pulmonary structural damage and have been correlated with lung function [23]. The Bhalla score is one of the most studied. We used only the final (total) Bhalla score based on its higher levels of reproducibility compared with each component of the score [24]. In addition, the available CT scans were acquired using different protocols, which could favour intra- and interobserver bias for fine details, thus further supporting the use of only the final score.

The combination of the F508del and Y1092X genetic mutations is expected to cause pancreatic insufficiency, as observed in these siblings [25]. However, the CF phenotype of the female patient was more severe since birth, and she had a markedly worse outcome than her brother. Siblings with CF often have different phenotypes and clinical courses, even in the presence of the same disease-causing genes, because the CF phenotype is affected not only by the CFTR genotype but also by environmental and other genetic factors [26]. MI, for example, occurs in almost 20% of CF patients and is more frequent with some CFTR mutations (F508del, G542X, W1282X, R553X, and G551D) but does not necessarily affect multiple siblings. This phenotypic variability has been partially attributed to modifier genes [27,28]. The female gender may also have influenced the outcome as a negative prognostic factor. Despite an equal prevalence of CF, females have a lower median life expectancy than males and a higher risk of respiratory infections [29]. Females acquire *P. aeruginosa* earlier and exhibit a more rapid decline of lung function once infected [30]. The mechanisms underlying this gender disparity has not been fully elucidated, but sex hormones play a role. Estrogen enhances the conversion of *P. aeruginosa* from the non-mucoid to mucoid phenotype, which is more resistant to antibiotics [31]. Studies also suggest that women have more frequent CF exacerbations during ovulation, a period of high estrogen levels. Harness-Bronley et al. [30] analysed a large cohort of patients from the United States Cystic Fibrosis Foundation Patient Registry and found that not only *P. aeruginosa* but also methicillin-susceptible *S. aureus*, methicillin-resistant *S. aureus*, *Haemophilus influenzae*, *A. xylosoxidans*, *Burkholderia cepacia*, *Aspergillus* species, and non-tuberculous mycobacterium are acquired by females at earlier ages, often even before puberty.

Like any study, ours has limitations. First, there was no overlap of periods of microbiological follow-up for the two siblings. Only a period of microbiological data was available that were obtained prior to objective clinical data, such as data related to CT scans and pulmonary function, thus limiting the potential for translational conclusions. Second, we were unable to determine the precise timing of the initial *A. xylosoxidans* infection. In addition, these sibling patients were also chronically colonized by *P. aeruginosa*, which may be a cause of poor outcome. Thus, more translational studies are needed to better understand the relationships between microbiological and clinical data and potentially improve patient management.

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Conflict of interest

The authors have no potential conflicts of interest to disclose.

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ANEXO A – Parecer consubstanciado do comitê de ética em pesquisa do HUPE/UERJ.

Plataforma Brasil - Ministério da Saúde

Hospital Universitário Pedro Ernesto/ Universidade do Estado do Rio de Janeiro

PROJETO DE PESQUISA

Título: Perfil de infecção por Achromobacter xylosoxidans e repercussão clínica em pacientes com fibrose cística acompanhados no Rio de Janeiro

Área Temática:

Pesquisador: Mônica de Cássia Firmida **Versão:** 2

Instituição: Hospital Universitário Pedro Ernesto/UERJ **CAAE:** 00716512.0.0000.5259

PARECER CONSUBSTANCIADO DO CEP

Número do Parecer: 36867

Data da Relatoria: 13/06/2012

Apresentação do Projeto:

Está bem estrurado.

Objetivo da Pesquisa:

Bem descrito.

Avaliação dos Riscos e Benefícios:

ok

Comentários e Considerações sobre a Pesquisa:

nada a declarar

Considerações sobre os Termos de apresentação obrigatória:

bada a declarar

Recomendações:

nenhuma

Conclusões ou Pendências e Lista de Inadequações:

nenhuma

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

Na data de emissão desse parecer estavam pendentes os pareceres de algumas co-participantes. As mesmas deverão ser apresentadas antes do início do projeto.

RIO DE JANEIRO, 14 de Junho de 2012

Assinado por:

WILLE OIGMAN

ANEXO B – Parecer consubstanciado do comitê de ética em pesquisa do IFF/Fiocruz.

INSTITUTO FERNANDES FIGUEIRA - IFF/ FIOCRUZ - RJ/ MS

PARECER CONSUBSTANCIADO DO CEP

Elaborado pela Instituição Coparticipante

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Perfil de infecção por Achromobacter xylosoxidans e repercussão clínica em pacientes com fibrose cística acompanhados no Rio de Janeiro

Pesquisador: Mônica de Cássia Firmida

Área Temática:

Versão: 2

CAAE: 00716512.0.3001.5269

Instituição Proponente: Hospital Universitário Pedro Ernesto/UERJ

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 593.386-0

Data da Relatoria: 19/06/2012

Apresentação do Projeto:

Esta pesquisa visa conhecer a frequência de infecção por A. xylosoxidans em pacientes com Fibrose cistica acompanhados no estado do Rio de Janeiro (RJ), descrever a frequência de multirresistência e avaliar o impacto desta infecção na progressão da
doença pulmonar. Serão estudados portadores de FC acompanhados nos dois principais centros de referência do RJ. A coorte de pacientes em
seguimento será descrita quanto a idade, sexo, etnia, critérios diagnósticos de FC e função pancreática exócrina. Com base em culturas de
espécimes respiratórias realizadas de rotina, serão descritos: perfil de colonização bacteriológica desta população, frequência de identificação do A.
xylosoxidans e fequência de multirresistência deste agente. A multirresistência será definida de acordo com os critérios propostos pelo Center of
Diseases Control and Prevention (CDC). Os pacientes com culturas positivas para A. xylosoxidans serão estratificados de acordo com o número de
exames positivos em uma, duas e três ou mais culturas positivas por ano, com intervalo mínimo de um mês entre elas. O peso de fatores como coinfecções
por outros agentes, antibióticos usados e frequência de internações no risco de infecção por A.

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xylosoxidans também será investigado.
Visando avaliar o impacto clínico, será selecionado um grupo de indivíduos infectados por A. xylosoxidans e colonizados crônicos por Pseudomonas
aeruginosa (P. aeruginosa) a ser comparado a um grupo controle de colonizados crônicos por P. aeruginosa sem A. xylosoxidans. O pareamento
dos grupos será feito por idade, gênero, VEF1 (±10%) e índice de massa corporal (IMC). Infectados por bactérias do complexo Burkholderia cepacia
serão excluídos. Evolutivamente, serão comparados: número de internações, antibióticos usados, IMC, VEF1 e alterações tomográficas (escore de
Bhalla) desde um ano antes da infecção até dois anos depois. Por fim, almeja-se propor um critério de colonização crônica por A. xylosoxidans.

Objetivo da Pesquisa:

Conhecer a frequência e o perfil da infecção por Achromobacter (Alcaligenes) xylosoxidans em uma coorte de pacientes com fibrose cística
acompanhados em dois centros de referência do estado do Rio de Janeiro e avaliar o impacto da colonização por este agente na evolução da
doença.

Avaliação dos Riscos e Benefícios:

Trata-se de um estudo descritivo, retrospectivo e não envolve riscos diretos ao paciente. A confidencialidade dos dados está prevista e conhecer os fatores de risco para esta infecção por Achromobacter xylosoxidans nesta população e seu impacto clínico pode ajudar no desenvolvimento de estratégias de prevenção e de controle da mesma. A definição de
critérios de colonização, ainda inexistentes, pode ser de grande auxílio não só para a assistência clínica, mas também para pesquisas futuras,
nacionais e internacionais.

Comentários e Considerações sobre a Pesquisa:

Estudo multicentrico ja apreciado e aprovado em 2 outros CEPs

Considerações sobre os Termos de apresentação obrigatória:

Adequados

Recomendações:

apresentar relatorio final

INSTITUTO FERNANDES FIGUEIRA - IFF/ FIOCRUZ - RJ/ MS

Continuação do Parecer: 593.386-0

Conclusões ou Pendências e Lista de Inadequações:

aprovar

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

Considerações Finais a critério do CEP:

RIO DE JANEIRO, 11 de Abril de 2014

Assinador por:
maria elisabeth lopes moreira
(Coordenador)

Este parecer reemitido substitui o parecer número 593386 gerado na data 03/07/2012 14:33:22, onde o número CAAE foi alterado de 00716512.0.0000.5259 para 00716512.0.3001.5269.

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