PROGRAMA DE PÓS-GRADUAÇÃO EM VIGILÂNCIA SANITÁRIA
INSTITUTO NACIONAL DE CONTROLE DE QUALIDADE EM SAÚDE
FUNDAÇÃO OSWALDO CRUZ

Catia Aparecida Chaia de Miranda

RELAÇÕES CLONAIS ENTRE *Pseudomonas aeruginosa* MULTIDROGA RESISTENTES DE ORIGEM CLÍNICA E DO EFLUENTE HOSPITALAR

Rio de Janeiro
2015

Catia Aparecida Chaia de Miranda

RELAÇÕES CLONAIS ENTRE *Pseudomonas aeruginosa* MULTIDROGA RESISTENTES DE ORIGEM CLÍNICA E DO EFLUENTE HOSPITALAR

Tese apresentada ao Programa de Pós-Graduação em Vigilância Sanitária do Instituto Nacional de Controle de Qualidade em Saúde da Fundação Oswaldo Cruz como requisito parcial para obtenção do título de Doutor em Vigilância Sanitária.

Orientadores: Maysa B. Mandetta Clementino
Ivano de Filippis

Rio de Janeiro

2015

Catalogação na fonte
Instituto Nacional de Controle de Qualidade em Saúde
Biblioteca

Miranda, Catia Aparecida Chaia de

Relações clonais entre *Pseudomonas aeruginosa* multidroga resistentes de origem clínica e do efluente hospitalar / Catia Aparecida Chaia de Miranda. Rio de Janeiro: INCQS /FIOCRUZ, 2015.

183 f., il.

Tese (Doutorado em Vigilância Sanitária) – Programa de Pós-Graduação em Vigilância Sanitária, Instituto Nacional de Controle de Qualidade em Saúde, Fundação Oswaldo Cruz. Rio de Janeiro, 2015.
Orientadoras: Maysa Beatriz Mandetta Clementino, Ivano De Filippis

1. Pseudomonas aeruginosa. 2. Genes MDR. 3. Resistência a Múltiplos Medicamentos. 4. Tipagem de Sequências Multilocus. 5. Resíduos de Serviços de Saúde. 6. Águas Residuais. I. Título

Clonal relationship between multidrug resistant Pseudomonas aeruginosa from clinical and hospital wastewater

Catia Aparecida Chaia de Miranda

RELAÇÕES CLONAIS ENTRE *Pseudomonas aeruginosa* MULTIDROGA RESISTENTES DE ORIGEM CLÍNICA E DO EFLUENTE HOSPITALAR

Tese apresentada ao Programa de Pós-Graduação em Vigilância Sanitária do Instituto Nacional de Controle de Qualidade em Saúde da Fundação Oswaldo Cruz como requisito parcial para obtenção do título de Doutor em Vigilância Sanitária.

Aprovada em: 16 / 12 / 2015

BANCA EXAMINADORA

Antonio Eugenio Castro Cardoso de Almeida
Instituto Nacional de Controle de Qualidade em Saúde - FIOCRUZ

Orlando Bonifácio Martins (Doutor)
Instituto de Bioquímica Médica - UFRJ

Alexander Machado Cardoso (Doutor)
Fundação Centro Universitário Estadual da Zona Oeste - UEZO

Maysa Beatriz Mandetta Clementino (Doutor) – Orientador
Instituto Nacional de Controle de Qualidade em Saúde - FIOCRUZ

Ivano De Filippis (Doutor) – Orientador
Instituto Nacional de Controle de Qualidade em Saúde - FIOCRUZ

Dedico este trabalho aos meus pais, Antonio (*in memoriam*) e Helena e ao meu filho Lucas.

Amor incondicional.

AGRADECIMENTOS

A Deus por se mostrar presente em todos os dias da minha vida. Sua presença é a força que eu preciso para seguir em frente.

Ao meu pai Antonio (*in memoriam*), meu tudo, que me ensinou o valor do estudo e a levantar sempre, desistir jamais.

À minha mãe Helena, meu porto seguro, por acreditar em mim e estar sempre ao meu lado.

Ao meu filho Lucas, meu GRANDE amigo, razão do meu viver. Obrigada por me dar força para continuar. Te amo!

Aos meus irmãos Carla e Toni, minha cunhada-irmã Luciana e aos meus sobrinhos Pedro, Luiza e Isabela pela linda família que formamos. Sem vocês seria difícil a caminhada.

À amiga e orientadora Maysa Beatriz Mandetta Clementino, que esteve presente em vários momentos da minha vida, me apoiando e mostrando o melhor caminho a seguir. Obrigada por tudo!!!

Ao meu orientador Ivano De Filippis, pelos ensinamentos, paciência e incentivo que foram fundamentais para a conclusão deste trabalho.

Ao amigo Antônio Eugênio, que me acompanhou e apoiou desde que eu entrei na Fiocruz. Obrigada por toda ajuda, pelos conselhos e momentos de descontração.

À Célia Romão por ter aceito fazer a revisão desta tese, participar das minhas bancas de Seminários e pela sua grande ajuda profissional, sempre que eu precisei.

Ao meu eterno Orientador Orlando Bonifácio Martins que com sua frase inesquecível: “perguntar não ofende”, me ensinou a refletir sobre os resultados e aprender que todo resultado é positivo, mesmo os que dão negativo. Obrigada por fazer parte da minha formação.

Ao amigo Alexander Cardoso que sempre aceitou os convites para participar das minhas bancas, pelas várias sugestões e idéias que sempre foram de grande importância.

Ao amigo Sérgio Ricardo por aceitar o convite para participar desta banca e por estar presente, sempre que eu preciso.

À amiga Jandira, “guru”, “anjo da guarda”, companheira de todas as horas, do cafezinho para relaxar. Obrigada pela grande ajuda nos momentos mais difíceis e pela sua amizade.

À amiga Talita Coelho, pelo companheirismo, confiança, paciência para me escutar e pela troca de conhecimentos que foram essenciais para que eu pudesse chegar até aqui.

À amiga Lúcia Werneck pelo apoio, proteção e palavras de carinho em todos os momentos.

Às amigas de todas as horas, do papo descontraído e muitas risadas: Claudia Andrade, Alice Batalha, Aline Azevedo, Carla Machado e Nathalia Caldeira.

Aos alunos do Setor de Bactérias de Referência: Juliana, Samara, Claudia Flores, Camila, Mariana, Pâmela, Bruna, Andressa, Renata e especialmente ao Kayo que sempre esteve pronto a me ajudar.

Às profissionais Verônica e Alessandra do Hospital Municipal Lourenço Jorge, que gentilmente, cederam as cepas clínicas utilizadas neste estudo.

A todos os professores que contribuíram para minha formação acadêmica, secretárias e coordenação da Pós-Graduação em Vigilância Sanitária.

Por fim, agradeço a todos que de alguma forma contribuíram para a realização deste trabalho.

Mesmo quando tudo parece desabar, cabe a mim decidir entre rir ou chorar, ir ou ficar, desistir ou lutar; porque descobri, no caminho incerto da vida, que o mais importante é o decidir.

Cora Coralina

RESUMO

O lançamento de efluentes hospitalares, sem pré-tratamento adequado, em corpos receptores, é motivo de preocupação devido à disseminação de poluentes químicos e biológicos aos corpos receptores. A presença de *Pseudomonas aeruginosa* multidroga resistentes está associada à sua ampla colonização nos hospitais e consequentemente nos efluentes e ao meio ambiente. O objetivo deste estudo foi investigar a relação clonal entre os isolados de *Pseudomonas aeruginosa* de origem clínica e do efluente hospitalar, e a possível associação clonal à diminuição da susceptibilidade aos antibióticos, principalmente em relação aos beta-lactâmicos. Cento e setenta e sete isolados de *P. aeruginosa* recuperados do hospital (n=136) e seu efluente (n=41), foram identificados fenotipicamente e certificados pela amplificação do gene 16S rRNA. Quanto ao perfil de susceptibilidade, os isolados foram analisados frente a 17 antibióticos pelo Método de disco difusão. Dos 41 isolados da ETEH, 88% foram resistentes à fosfomicina, seguidos por ticarcilina/ácido clavulânico (71%) e ceftriaxona (63%). Os 136 isolados clínicos apresentaram maior valor de resistência a fosfomicina (100%), seguido por cefotaxima (79%) e ceftriaxona (76%). A produção de beta-lactamase foi verificada por meio de cultivo em Chromoagar – ESBL e pelo teste de Carba NP. Os genes codificadores de ESBL (*bla*$_{PER}$, *bla*$_{VEB}$, *bla*$_{SHV}$, *bla*$_{CTX\text{-}M\text{-}1}$, *bla*$_{CTX\text{-}M\text{-}2}$, *bla*$_{CTX\text{-}M\text{-}8}$, *bla*$_{CTX\text{-}M\text{-}9}$, *bla*$_{CTX\text{-}M\text{-}25}$, *bla*$_{TEM}$ e *bla*$_{GES}$), de MBL (*bla*$_{IMP}$, *bla*$_{VIM}$, *bla*$_{SPM}$ e *bla*$_{NDM}$) e de KPC (*bla*$_{KPC}$) foram detectados pela PCR. Cento e onze isolados (63%) resistentes a pelo menos um antibiótico pertencente a três ou mais classes foram classificados como multirresistentes. Destes, 37 (33%) foram classificados como Multidroga Resistentes (MDR) e 74 (67%) Extensivamente Droga Resistentes (XDR). A relação genética dos isolados foi determinada através da ERIC-PCR e MLST, agrupando em 69 perfis distintos (HMLJ (n=49; ETEH (n=20)) e 51 ST (HMLJ (n=36); ETEH (n=15)), respectivamente. O ST 244 foi o único presente nos dois ambientes analisados. Os dados gerados a partir da árvore de MST, com isolados dos dois ambientes, mostraram que o ST595 e o ST1941 compartilham cinco alelos idênticos com o ST244 e devem ser incluídos no Complexo Clonal 244 (CC244). O CC244 demonstrou grande potencial patogênico deste clone por carrear cepas MDR e XDR de diferentes fontes, tais como clínica, água e efluente hospitalar. Nossos resultados nos permitem concluir que o tratamento terciário dispensado ao efluente hospitalar

não foi totalmente eficiente, uma vez que foi demonstrado um aumento de linhagens XDR no efluente tratado. A grande diversidade genética de *Pseudomonas aeruginosa* recuperadas do hospital e seu efluente, constantemente liberadas no sistema aquático, contribui com a disseminação de genes de resistência entre microrganismos que compartilham o mesmo meio. Estas linhagens contribuem para a disseminação de microrganismos e genes de resistência podendo gerar impactos negativos ao meio ambiente e à saúde humana.

Palavras-chave: *Pseudomonas aeruginosa*. Multidroga resistente. Ambiente hospitalar. Efluente hospitalar. Complexo Clonal. Multilocus Sequence Typing

ABSTRACT

The disposal of hospital waste without adequate pre-treatment in the environment is a concern due to the spread of chemical and biological pollutants to receiving water streams. Distribution of multidrug resistant *Pseudomonas aeruginosa* is associated with a broad colonization in hospitals and subsequent transmission to the effluents and the environment. The aim of this study was to investigate the clonal relationship among *Pseudomonas aeruginosa* isolates from clinical origin and hospital sewage, and the potential clonal association with decreased susceptibility to antibiotics, especially for β-lactams. One hundred seventy-seven isolates of *P. aeruginosa*, recovered from a hospital (n=136) and its HWTP (n=41), were identified phenotypically and confirmed by a*mplification of the 16S rRNA* gene. Susceptibility profiles of isolates were analyzed against 17 antibiotics by disk diffusion Method. Of the 41 isolates of HWTP, 88% were resistant to fosfomycin, followed by ticarcillin/clavulanic acid (71%) and ceftriaxone (63%). One hundred thirty-six clinical isolates had greater value of resistance to fosfomycin (100%), followed by cefotaxime (79%) and ceftriaxone (76%). Production of beta-lactamase was investigated by screening in Chromoagar - ESBL and Carba NP test. Genes encoding ESBL (bla_{PER}, bla_{VEB}, bla_{SHV}, $bla_{CTX\text{-}M\text{-}1}$, $bla_{CTX\text{-}M\text{-}2}$, $bla_{CTX\text{-}M\text{-}8}$, $bla_{CTX\text{-}M\text{-}9}$, $bla_{CTX\text{-}M\text{-}25}$, bla_{TEM} and bla_{GES}), MBL (bla_{IMP}, bla_{VIM}, bla_{SPM} and bla_{NDM}) and KPC (bla_{KPC}) were screened by PCR. One hundred and eleven isolated (63%) were resistant to at least one antibiotic belonging to three or more classes and were classified as multiresistant. Thirty seven (33%) were classified as MDR and 74 (67%) XDR. Genetic relationships of the isolates were determined by ERIC-PCR and MLST, clustering into 69 distinct profile (Hospital (n=49; HWTP (n=20)) and 51 ST (Hospital (n=36); HWTP (n=15)), respectively. Sequence Type 244 was the only ST present in both environments analyzed. Data generated from the MLST tree with isolates from the two environments showed that ST595 and ST1941 share five identical alleles with ST244 and should be included in Clonal Complex 244 (CC244). CC244 showed a great pathogenic potential carrying MDR and XDR strains from different sources such as clinical, water and hospital effluent. Our findings allow us to conclude that the tertiary treatment dispensed to the hospital effluents was not very efficient, because of the high proportion of XDR strains found in treated effluent. The great genetic diversity of *Pseudomonas aeruginosa* recovered from the hospital and its effluent, constantly

released into the water system, contributes to the spread of resistance genes between organisms sharing the same environment. These strains contribute to the spread of microorganisms and resistance genes, which may generate negative impacts on the environment and human health.

Key words: *Pseudomonas aeruginosa*. Multidrug-resistant. Hospital environment. Hospital effluent. Clonal Complex. Multilocus Sequence Typing

LISTA DE ILUSTRAÇÕES

LISTA DE TABELAS

LISTA DE SIGLAS

ABNT - Associação Brasileira de Normas Técnicas
*acs*A - Acetil coenzima A sintetase
AIM - Australia Imipenemase
AmpC - adenosina 3',5'-monofosfato cíclico
ANVISA - Agência Nacional de Vigilância Sanitária
*aro*E - Chiquimato desidrogenase
ATCC - American Type Culture Collection
BGN-NF - Bacilos Gram-negativos não-fermentadores
BLAST - Basic Local Alignment Search Tool
Carba NP - Carbapenemase Nordmann-Poirel
CC - Complexo Clonal
CCIH - Comissão de Controle de Infecção Hospitalar
CEDAE - Companhia Estadual de Águas e Esgotos
CLSI - Clinical and Laboratory Standard Institute
CNES - Cadastro Nacional de Estabelecimentos de Saúde
CONAMA - Conselho Nacional de Meio Ambiente
CPI - Comissão Parlamentar de Inquérito
CTI - Centro de Terapia Intensiva
CTX - Cefotaximase
DIM - Dutch imipenemase
DNA - Deoxyribonucleic Acid
dNTPs - Deoxinucleotídeo trifosfato
ERIC - Enterobacterial Repetitive Intergenic Consensus
ESBL - Extended Spectrum Beta-lactamase
ETEH - Estação de Tratamento de Esgoto Hospitalar
FUNASA - Fundação Nacional de Saúde
GES - Guiana Extended Spectrum
GIM - German Imipenemase
*gua*A - GMP sintase
HMLJ - Hospital Municipal Lourenço Jorge
IMP - Imipenemase

INCQS - Instituto Nacional de Controle de Qualidade em Saúde
IRAS - Infecções Relacionadas à Assistência à Saúde
KHM - Kyorin University Hospital Metallo Beta-lactamase
KPC - *Klebsiella pneumoniae* Carbapenemase
LMR - Laboratório de Microrganismos de Referência
LPSN - List of Prokaryotic Names with Standing in Nomenclature
MBL - Metalo-Beta-Lactamase
MDR - Multidroga Resistente
MEGA - Molecular Evolutionary Genetics Analysis
MLST - Multilocus Sequence Typing
MST - Minimum Spanning Tree
*mut*L - DNA de proteínas de reparo “mismatch”
NBR - Norma Brasileira Regulamentadora
NCBI - National Center for Biotechnology Information
NDM - New Delhi Metallo beta-lactamase
NI - Não Informado
*nuo*D - NADH desidrogenase I cadeia C, D
OD - Oxigênio Dissolvido
OMS - Organização Mundial de Saúde
ONU - Organização das Nações Unidas
pb - pares de base
PCR - Reação em Cadeia da Polimerase
PER - *Pseudomonas* Extended Resistant
PFGE - Pulsed Field Gel Electrophoresis
pH - potencial de hidrogênio
PM - Peso Molecular
*pps*A - Fosfoenolpiruvato Sintase
RAPD - Random Amplified Polymorphic DNA
RDC - Resolução da Diretoria Colegiada
RNA - Ácido ribonucleico
RSS - Resíduos de Serviços de Saúde
RSU - Resíduos Sólidos Urbanos
SHV - Sulfhydryl reagente variable
SIM - Seul Imipenemase

SINVSA - Subsistema Nacional de Vigilância em Saúde Ambiental

SMAC - Secretaria Municipal de Meio Ambiente

SNGPC - Sistema Nacional de Gerenciamento de Produtos Controlados

SNVS - Sistema Nacional de Vigilância Sanitária

SPM - São Paulo Metalo-beta-lactamase

ST - Sequence Type

SVS - Secretaria de Vigilância em Saúde

TEM - Temoneira

*trp*E - Sintetase Anthralite componente I

UPGMA - Unweighted Pair Group Method with Arithmetic Average

UTI - Unidade de Terapia Intensiva

VEB - Vietnam Extended Spectrum Beta-lactamase

VIM - Verona Imipenemase

XDR - Extensivamente Droga Resistente

LISTA DE ABREVIATURAS

AMI - amicacina
Art. - artigo
ATB - antimicrobiano
AZT - aztreonam
CAZ - ceftazidima
CIP - ciprofloxacina
°C - grau celsius
cm - centímetro
COL - colistina
CPM - cefepima
CTR - ceftriaxona
CTX - cefotaxima
FOSFO - fosfomicina
GEN - gentamicina
h - hora (s)
IMI - imipenema
Kb - kilo bases
kHz - quilohertz
L - litro
LEV - levofloxacina
MEM - Meropenema
mg - miligrama
mL - mililitro
mM - milimolar
mm^3 - milímetro cúbico
nº - número
POL - polimixina
ppm - parte por milhão
PTZ - piperacillina/tazobactam
Sm/cm - milisiemens por centímetro
TOB - tobramicina

TIM - ticarcillina/ácido clavulânico

VF - vermelho de fenol

V - volts

$ZnSO_4$ - sulfato de zinco

W - watt

µg - micrograma

µM - micromolar

µm - micrômetro

β - beta

SUMÁRIO

1. INTRODUÇÃO

1.1. VIGILÂNCIA EM SAÚDE AMBIENTAL

A temática ambiental integra, atualmente, a agenda nacional e internacional, cabendo não só ao poder público, mas também à sociedade, a conservação do meio ambiente (RIBEIRO; BERTOLOZZI, 2004). Os sérios problemas ambientais que afetavam o mundo levaram à convocação pela Assembleia Geral da Organização das Nações Unidas (ONU), em 1968, da Conferência das Nações Unidas sobre o Meio Ambiente Humano, em junho de 1972 em Estocolmo. Essa Conferência chamou a atenção das nações para o fato de que a ação humana estava causando séria degradação da natureza e criando severos riscos para o bem-estar e para a própria sobrevivência da humanidade (BARCELLOS; QUITÉRIO, 2006).

Em 1988, a ONU aprovou uma Resolução determinando a realização, até 1992, de uma Conferência sobre o meio ambiente e desenvolvimento para avaliar como os países haviam promovido a proteção ambiental desde a Conferência de Estocolmo de 1972. A cidade do Rio de Janeiro foi sede desse evento por duas vezes consecutivas, a ECO92, em 1992 e a RIO+20 em 2012 (RIBEIRO; BERTOLOZZI, 2004) onde os Países renovaram seus compromissos com o desenvolvimento sustentável na Rio+20 – prometendo promover um futuro econômico, social e ambientalmente sustentável para o nosso planeta e para as gerações do presente e do futuro. Países também reafirmaram os princípios enunciados na Cúpula da Terra de 1992 e em diversas conferências subsequentes sobre desenvolvimento sustentável.

No Brasil, no setor saúde, tradicionalmente a vigilância sanitária tem se incumbido das ações sobre o ambiente, embora tais ações estejam limitadas, via de regra, ao saneamento básico, desde sua origem. Assim, a questão ambiental, como parte das condições de saúde e como objeto das políticas públicas, são temas inseridos na esfera da vigilância sanitária. De acordo com o Ranking do Saneamento do Instituto Trata Brasil (2014), 2,5 bilhões de pessoas (36%) viviam sem

saneamento adequado, 71% eram de áreas rurais. A cobertura de saneamento adequado aumentou de 49% em 1990 para 64% em 2012. Entre 1990 e 2012, quase dois bilhões de pessoas ganharam acesso ao saneamento.

Em junho de 2003 foi criada a Secretaria de Vigilância em Saúde (SVS) no âmbito do Ministério da Saúde e dentre as competências inclui-se a Coordenação da Gestão do Subsistema Nacional de Vigilância em Saúde Ambiental (SINVSA). Esse sistema é definido como um conjunto de ações que proporciona o conhecimento e a detecção de qualquer alteração nos fatores determinantes e condicionantes do meio ambiente que interferem na saúde humana, com a finalidade de identificar as medidas de prevenção e controle dos fatores de risco ambientais relacionados às doenças ou outros agravos à saúde (BRASIL, 2002). Dentre seus objetivos, está a adoção de ações integradas, de modo a exercer a vigilância desses fatores de risco ambientais que possam vir a afetar a saúde da população, estabelecendo parâmetros, identificando e intervindo nos fatores que levem a doenças e outros agravos, tendo em vista a eliminação do risco, o controle e a recuperação do meio ambiente (BRASIL, 2010a).

1.2. VIGILÂNCIA SANITÁRIA

Vigilância, em saúde Pública, é um termo relativamente novo; é, no conceito de Alexander Langmuir (1963), a observação contínua da distribuição e da tendência de doenças, mediante a coleta sistemática e a avaliação de dados de morbidade e mortalidade, assim como de outros dados relevantes, e a regular disseminação dessas informações. Vigilância é resultado de progresso. Já “sanitário”, curiosamente é um adjetivo que nos remete ao passado, à época dos sanitaristas e do sanitarismo.

Vigilância Sanitária é o resultado de duas vertentes históricas, uma antiga e outra moderna e também o testemunho de duas posturas diante de problemas de saúde, uma elitista autoritária, outra democrática e baseada no diálogo da população. Surgiu para prevenir ou diminuir riscos provocados por problemas

sanitários decorrentes do meio ambiente, ou originados de doenças infecto contagiosas (BUENO, 2005).

A Lei 6360, emitida em 1976 e que ficou conhecida justamente pelo nome de Lei da Vigilância Sanitária, dispõe sobre a vigilância sanitária de medicamentos, drogas, insumos farmacêuticos e saneantes, entre outros produtos. Somente a partir dessa lei foi estabelecida a exigência de apresentação de receita médica na venda de determinados medicamentos. A Medida Provisória nº 1.791/98 foi aprovada em 13 de janeiro de 1999, originando a Lei nº 9.782/99, de criação da Agência Nacional de Vigilância Sanitária (ANVISA), promulgada em 26 de janeiro do mesmo ano. A ANVISA ficou constituída como uma autarquia, sob regime especial, vinculada ao Ministério da Saúde. Em 26 de abril de 2000, a Agência teve sua sigla modificada para ANVISA pela medida provisória nº 2.134-29.

A organização da Vigilância Sanitária no Brasil foi modificada com a edição da Lei 9.782 de 1999, que criou a Agência Nacional de Vigilância Sanitária (ANVISA) – que substituiu a antiga Secretaria de Vigilância Sanitária integrante do Ministério da Saúde – e definiu o Sistema Nacional de Vigilância Sanitária (SNVS), entrando em consonância com os ordenamentos jurídicos que organizam o sistema de saúde brasileiro, e se inserindo nas estratégias para se enfrentar os problemas da época, como a crise dos medicamentos falsificados no país (DALLARI, 2001; COSTA, 2000; IVAMA; MELCHIOR, 2007).

De acordo com a Lei federal n° 8.080, de 19 de setembro de 1990 é função do Estado prover as condições necessárias para garantia da saúde da população e do meio ambiente, executando ações que reduzam o risco de doenças e outros agravos (BRASIL, 1990). Atualmente, a Vigilância Sanitária é conceituada como um conjunto de ações capazes de eliminar, diminuir ou prevenir riscos à saúde e de intervir nos problemas sanitários decorrentes do meio ambiente, da produção e da circulação de bens e da prestação de serviços de interesse da saúde, abrangendo: 1 - o controle de bens de consumo que, direta ou indiretamente, se relacionem com a saúde, compreendidas todas as etapas de processo, da produção ao consumo; 2 - o controle da prestação de serviços que se relacionam direta ou indiretamente com a saúde. No que se referem ao meio ambiente, os recursos naturais representam uma relevante parcela, em especial a água (BRASIL, 1990).

As civilizações sempre identificaram a água como fonte de vida e energia e os povoados humanos sempre se estabeleceram próximos aos locais que fornecessem este recurso natural (BUSTOS, 2003). Além da preocupação com a água, a humanidade se deparou com outras necessidades, dentre elas a busca de soluções para os problemas de saúde. Para isso, construíram locais de assistência médica. Com o início da assistência hospitalar, certamente houve o início de geração de Resíduos de Serviços de Saúde (RSS), entretanto somente há pouco mais de uma década este vem se tornando um assunto bastante discutido, devido ao grande impacto ocorrido no campo da infecção hospitalar e no meio ambiente (RIBEIRO FILHO, 2000).

1.3. RESÍDUOS DE SERVIÇOS DE SAÚDE

A geração de resíduos pelas diversas atividades humanas constitui-se atualmente em um grande desafio a ser enfrentado pelas administrações municipais, sobretudo nos grandes centros urbanos. A partir da segunda metade do século XX, com os novos padrões de consumo da sociedade industrial, a produção de resíduos vem crescendo continuamente em ritmo superior à capacidade de absorção da natureza (BRASIL, 2006).

Os dados do Panorama dos resíduos sólidos no Brasil, divulgado pela Associação Brasileira de Empresas de Limpeza Pública e Resíduos Especiais (ABRELPE, 2013) mostram que no Brasil, a cada dia são geradas 209.280 toneladas de resíduos sólidos urbanos (RSU). Esse total é correspondente à soma dos resíduos sólidos urbanos gerados em todo o país. Deste total, apenas 58,3 % tiveram uma destinação final adequada e 41,74% uma destinação inadequada seguindo para lixões ou aterros controlados, que do ponto de vista ambiental pouco se diferenciam dos lixões, pois não possuem o conjunto de sistemas necessários para a proteção do meio ambiente e da saúde pública. Segundo a estimativa da ANVISA os resíduos de serviços de saúde (RSS) respondem por 1% do total. É um desafio para a comunidade, principalmente para os envolvidos diretamente com

esses resíduos, que tem a competência de minimizar e gerenciar adequadamente o “lixo”, a fim de se evitar contaminação e impactos ao meio ambiente.

É importante salientar que os RSS não se restringem apenas aos resíduos gerados nos hospitais, mas também a todos os demais estabelecimentos geradores de resíduos de saúde, a exemplo de postos de Saúde, laboratórios patológicos e de análises clínicas, clínicas veterinárias, centros de pesquisas, laboratórios, banco de sangue, consultórios médicos, odontológicos e similares.

A Resolução nº 05/93 do Conselho Nacional do Meio Ambiente - CONAMA, traz no seu bojo o conceito de resíduos sólidos definido pela NBR 10.004/87 da ABNT, que revela: "Resíduos nos estados sólidos e semissólidos, que resultam de atividades da comunidade de origem: industrial, doméstica, hospitalar, comercial, agrícola, de serviços e de varrição. Ficam incluídos nesta definição os lodos provenientes de sistemas de tratamento de água, aqueles gerados em equipamentos e instalações de controle de poluição, bem como determinados líquidos cujas particularidades tornem inviável seu lançamento na rede pública de esgotos ou corpos d'água, ou exijam para isso soluções técnica e economicamente inviáveis, em face à melhor tecnologia disponível". Sendo importante salientar que quando se diz “resíduo sólido” nem sempre se pode associar aquele a um estado sólido (BRASIL, 1993).

A grande preocupação atual a respeito do gerenciamento e destino dos resíduos provenientes dos serviços de saúde é expressa através da promulgação da resolução do CONAMA nº 358/05 que classifica os RSS em cinco grupos: A (potencialmente infectantes), B (químicos), C (rejeitos radioativos), D (comuns) e E (perfuro-cortantes) e estabelece os critérios mínimos necessários para o seu correto tratamento e disposição final (BRASIL, 2005).

Ainda como resíduos de serviços de saúde se encontram os resíduos líquidos que são eliminados através dos sistemas de esgoto oriundos das unidades de saúde, que também são alvo de preocupação da ANVISA em face aos riscos correlacionados com a saúde pública e o meio ambiente (BRASIL, 2004).

No Brasil, a grande maioria dos municípios não possui sistema de tratamento de esgotos. Em um esgoto predominantemente doméstico, 75% dos sólidos em suspensão e 40% dos sólidos dissolvidos são de natureza orgânica (PIVELI; KATO, 2006).

De acordo com Silveira e Monteggia (2003), as atividades desenvolvidas nos serviços de saúde resultam na geração de diferentes tipos de resíduos, sólidos e líquidos, e os impactos gerados pelos mesmos dependem da forma de gerenciamento dentro e fora da instituição, podendo provocar modificações no meio ambiente, mesmo quando presentes em concentrações mínimas.

Estudos têm demonstrado que efluentes hospitalares apresentam níveis mais elevados de bactérias entéricas resistentes aos antimicrobianos do que efluentes derivados de outras fontes e, que a concentração de antimicrobianos na água que recebe o esgoto hospitalar, também é superior, criando um ambiente com forte pressão seletiva (FUENTEFRIA et al, 2008). Ainda, a constituição genética destes microrganismos pode ser alterada por meio dos efeitos diretos e indiretos das substâncias constituintes dos efluentes hospitalares, levando ao aparecimento de bactérias multirresistentes aos antibióticos (DODD, 2012).

Dados da Organização Mundial de Saúde (OMS) apontam que mais de 50% das prescrições de antimicrobianos no mundo são inadequadas. No Brasil, o consumo de antibióticos chega a 40% dos medicamentos comercializados, com mais de 70 milhões de unidades comercializadas, segundo relatório do Instituto IMS Health. O uso indiscriminado desses antibióticos fez com que a ANVISA adotasse medidas mais restritivas para fortalecer a política de uso racional de antimicrobianos contribuindo para a redução da resistência bacteriana na população, através da implementação da RDC 44/2010 (BRASIL, 2010b), atualizada pela RDC 20/2011 (BRASIL, 2011). Esta nova resolução determinou que os antibióticos vendidos nas farmácias e drogarias do país apenas poderão ser entregues ao consumidor mediante receita de controle especial em duas vias, e a partir de janeiro de 2013 somente poderão ser comercializados mediante escrituração obrigatória no Sistema Nacional de Gerenciamento de Produtos Controlados (SNGPC). Assim sendo, o tratamento e o destino dado ao esgoto hospitalar são questões primordiais na prevenção da disseminação da resistência.

A Resolução 306 de Dezembro de 2004, da Agência Nacional de Vigilância Sanitária (BRASIL, 2004), estabelece que os resíduos líquidos oriundos de estabelecimentos de saúde devem ser tratados antes do seu lançamento em um corpo d’água ou na rede coletora de esgoto quando esses locais não são atendidos por um serviço de tratamento de esgoto coletivo.

1.4. GÊNERO *Pseudomonas*

O gênero *Pseudomonas*, descrito por Migula (1894), compreende organismos muito versáteis metabolicamente, capazes de utilizar uma grande variedade de compostos orgânicos como fonte nutricional (LATOUR; LEMANCEAU, 1997). Consequentemente, eles estão distribuídos nos diferentes ambientes como solos e água, sedimentos, amostras clínicas, planta, fungos e espécimes animais, desertos e etc (PEIX; RAMÍREZ-BAHENA; VELÁZQUEZ, 2009).

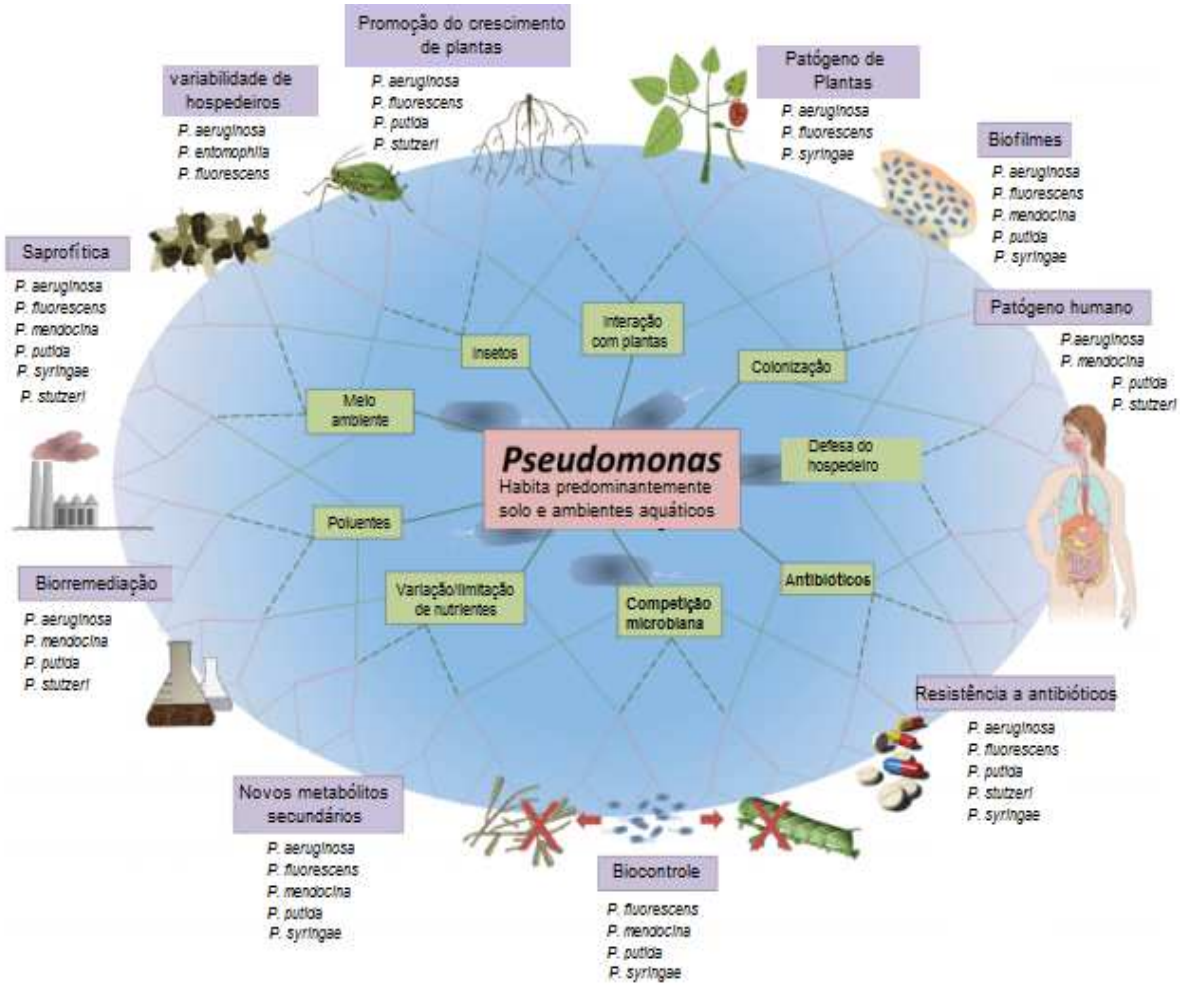
Desde sua descoberta, o gênero foi submetido a numerosas revisões taxonômicas, envolvendo não só o número de espécies incluídas, mas também os critérios utilizados para sua definição e delimitação. A variedade de parâmetros utilizados revela a dificuldade e os esforços para a distinção das espécies deste gênero e incluem: a análise da morfologia, da estrutura e da composição da parede celular, do tipo de pigmento, das características nutricionais e metabólicas, da susceptibilidade a diferentes compostos, da patogenicidade para diferentes organismos, da estrutura antigênica e das características genéticas e ecológicas (PALLERONI, 2005; PEIX; RAMÍREZ-BAHENA; VELÁZQUEZ, 2009).

Atualmente, o gênero *Pseudomonas* conta com 218 espécies e 18 subespécies (“List of prokaryotic names with standing in nomenclature”, LPSN; http://www.bacterio.cict.fr; acessado em 18 de novembro de 2015). As espécies do gênero *Pseudomonas* são bastonetes Gram negativos, retos ou ligeiramente curvos, aeróbios estritos, de tamanho médio com aproximadamente 1,5-5,0 X 0,5-1,0 µm. São estritamente aeróbias, oxidativas, catalase positivas, oxidase positiva, sendo a maioria móvel, através de um ou vários flagelos polares. Algumas espécies produzem pigmentos solúveis e a maioria cresce no meio de MacConkey (GOMES, 2008).

As espécies do gênero *Pseudomonas* se destacam pela grande versatilidade metabólica e fisiológica, que permite a colonização em diversos ambientes terrestres e aquáticos (PALLERONI, 1992), e são de grande interesse devido a sua importância como agente causador de doenças em plantas e animais, bem como seu potencial em aplicações biotecnológicas (SILBY et al, 2011) (Figura 1).

Até o momento já foram sequenciados 98 genomas completos e 1447 genomas incompletos de espécies do gênero de *Pseudomonas*, com um total de 5561998 genes. Em relação à espécie *P. aeruginosa*, já foram sequenciados 26 genomas completos e 970 genomas incompletos (The *Pseudomonas* Genome Database; http://beta.pseudomonas.com; acessado em 21 de julho de 2015). As linhagens de *P. aeruginosa* apresentam genomas relativamente maiores que aqueles da maioria das bactérias sequenciadas, variam de 5,5 a 7Mb (KLOCKGETHER et al, 2011) e possuem uma complexidade genética que permite que esta bactéria se adapte a diversos ambientes e cresça em condições menos favoráveis.

Figura 1. A diversidade funcional e ambiental de *Pseudomonas* spp.



O ancestral comum do gênero *Pseudomonas* encontrou uma variedade de ambientes bióticos e abióticos que levou à evolução de diversas características e estilos de vida com significante sobreposição entre as espécies. Adaptado de SILBY e colaboradores (2011).

1.4.1. *Pseudomonas aeruginosa*

A espécie *P. aeruginosa* é a mais importante do gênero, tanto pela frequência em infecções diversas quanto pelos seus fatores de virulência. Microscopicamente, este microrganismo é observado na forma de bacilos Gram negativos, diferenciados por meio de provas bioquímicas, formação de pigmentos, sensibilidade a antibióticos, números, localização dos flagelos, com crescimento ótimo entre 37 a 42°C, capazes de converter o nitrato a nitrito e/ou gás nitrogênio e produtores de pigmentos difusíveis em meios de cultura sólidos,

incluindo pioverdina (verde), piocinina (azul), piorrubina (vermelho) e piomelanina (marrom) (KISKA; GILLIGAN, 1999). Facilmente cultivadas nos meios de cultivo comuns (ágar nutriente, ágar caseína soja, ágar Mueller Hinton, ágar sangue etc.). Os cultivos produzem um odor característico, apresentando coloração esverdeada.

Considerado um organismo saprófito difundido na natureza, particularmente em ambientes úmidos (água, solo, plantas e esgoto), e dotado de pequeno potencial patogênico (PEIX; RAMÍREZ-BAHENA; VELÁZQUEZ, 2009). No entanto, devido a sua habilidade de sobreviver em materiais inertes e sua resistência a muitos antissépticos e antibióticos, *P. aeruginosa* tem se tornado um importante e frequente patógeno nosocomial (DUBOIS et al, 2001) e um dos principais microrganismos encontrados em efluentes hospitalares (GALES; REIS; JONES, 2001).

Embora raramente possa causar patologias em indivíduos sadios, essa bactéria é uma grande ameaça a pacientes imuno-comprometidos (DUBOIS et al, 2001). Este organismo é responsável por uma ampla gama de infecções relacionadas à assistência à saúde (IRAS), tais como pneumonia, infecções do trato urinário, ou bacteremia.

A crescente detecção de infecções hospitalares causadas por cepas multirresistentes de *P. aeruginosa* tem sido motivo de grande preocupação. Diversos mecanismos estão envolvidos na resistência aos antimicrobianos expressa por esses microrganismos.

1.5. *Pseudomonas aeruginosa* NO AMBIENTE HOSPITALR

Pseudomonas aeruginosa é uma bactéria amplamente distribuída no ambiente hospitalar, particularmente em áreas úmidas, sendo que, raramente, ocorre transmissão entre pessoas. Muito difundida na natureza, desperta particular interesse por ser um dos organismos mais encontrados nas infecções hospitalares, principalmente em pacientes imuno-comprometidos (De VOS et al, 1997). Tais infecções apresentam elevada morbidade e mortalidade e estão no centro das preocupações da comunidade científica (CEZÁRIO et al, 2004).

A ocorrência das infecções causadas por microrganismos resistentes aos antimicrobianos, constitui um problema mundial de saúde pública. Sabe-se que os hospitais, em geral, são caracterizados como ambientes onde é exercida uma grande pressão seletiva devido à enorme variedade de substâncias químicas utilizadas, como parte de programas de controle de infecções hospitalares. A associação dessas substâncias e microrganismos exerce forte pressão seletiva que resulta na seleção de bactérias multirresistentes (YANG et al, 2009).

Historicamente, *Staphylococcus* spp., *P. aeruginosa* e *Escherichia coli* são os agentes mais importantes das infecções hospitalares. Pneumonia nosocomial, infecções do trato urinário, infecções de feridas cirúrgicas e infecções da corrente sanguínea têm causado a maioria das doenças e óbitos em pacientes hospitalizados (MASEDA et al, 2011).

O tratamento das infecções por *P. aeruginosa* é, muitas vezes, difícil devido à resistência intrínseca e adquirida deste organismo aos antimicrobianos. Infecções por este microrganismo multirresistente a drogas estão aumentando em todo o mundo. Embora a definição de multirresistência seja variável, frequentemente associa-se à resistência a fluorquinolonas, cefalosporinas de amplo espectro, carbapenêmicos e aminoglicosídeos (NORDMANN et al, 2010).

Frequentemente, isolados de *P. aeruginosa* apresentam um amplo espectro de resistência, podendo ser resistentes a diferentes classes de agentes antimicrobianos, inclusive contra cefalosporinas de terceira e quarta gerações e carbapenêmicos (como imipenema e meropenema) (FUENTEFRIA et al, 2008).

Os dois principais fatores envolvidos no desenvolvimento da resistência aos antibióticos em bactérias são a pressão seletiva e a presença de genes de resistência (WITTE, 2000). Os genes que codificam resistência aos antimicrobianos podem estar localizados nos plasmídeos, *transposons* e *integrons* permitindo a sua transferência entre diferentes gêneros e espécies além das espécies com resistência intrínseca.

A grande preocupação em detectar amostras clínicas multirresistentes está no fato de que alguns genes de resistência estão inseridos na forma de cassetes de genes em elementos chamados de integrons. Eles são definidos como elementos genéticos que podem conter um ou mais genes de resistência inseridos por meio de um sistema de recombinação sítio-específico (STOKES; HALL, 1989). Os cassetes

conhecidos acomodam genes que conferem resistência a antibióticos, assim como genes de resistência a antissépticos e desinfetantes (ROMÃO et al, 2011).

Infecções, causadas por agentes etiológicos amplamente resistentes aos antimicrobianos, representam um dos grandes desafios atuais da saúde pública, acarretando em altas taxas de morbimortalidade, aumento no tempo de internação e nos gastos do sistema de saúde (NEIDELL et al, 2012).

1.6. RESISTÊNCIA NATURAL E ADQUIRIDA AOS ANTIMICROBIANOS

A resistência bacteriana tem crescido significativamente nos últimos anos. Entre as bactérias Gram-negativas, a *P. aeruginosa* apresenta maior facilidade de desenvolvimento de resistência a antibióticos. Esse microrganismo pode apresentar resistência natural ou adquirida a grande número de antibióticos utilizados na prática clínica (YONEDA et al, 2005). O intercâmbio de material genético, que ocorre de forma natural intra ou interespécies entre os bacilos Gram-negativos, é apontado como um dos responsáveis pela aquisição de determinantes de resistência (NEVES, 2011).

A resistência intrínseca é natural e evolutivamente conservada nos microrganismos, resultante da genética, estrutura e fisiologia do microrganismo. No entanto, este tipo de resistência é rara e está associada apenas a alguns indivíduos da população bacteriana. A resistência adquirida é resultado de mutações e transferência horizontal de genes (LORIAN, 2005).

Entre os antibióticos mais empregados na prática médica estão os beta-lactâmicos. Essa classe de antimicrobianos compreende todos os agentes antimicrobianos que contêm um anel beta-lactâmico na sua estrutura molecular. Essa classe inclui as penicilinas, cefalosporinas, monobactâmicos e carbapenêmicos. O mecanismo de ação desses antibióticos compreende a inibição da síntese da parede celular das bactérias e eles estão entre os mais frequentemente prescritos em todo o mundo (PITOUT et al, 2005). Um dos mecanismos mais importantes de resistência aos antibióticos beta-lactâmicos é a produção de beta-lactamases, enzimas bacterianas capazes de hidrolisar o anel

beta-lactâmico presente nestes antibióticos, tornando-os inativos (WILKE; LOVERING; STRYNADKA, 2005; BABIC; HUJER; BONOMO, 2006).

A capacidade ou não das beta-lactamases em conferir resistência irá depender da quantidade de enzima produzida, da habilidade desta enzima em hidrolisar o antimicrobiano em questão, e da velocidade com que o beta-lactâmico penetra pela membrana celular externa da bactéria (LIVERMORE, 2001).

A beta-lactamase cromossomal AmpC desempenha um importante papel na resistência antimicrobiana em isolados clínicos de *P. aeruginosa* uma vez que sua expressão pode ser induzida na presença de alguns antimicrobianos beta-lactâmicos diminuindo sua sensibilidade aos mesmos (LIVERMORE; WOODFORD, 2006). Estas enzimas têm a capacidade de hidrolisar cefalosporinas de primeira, segunda e terceira geração, mas não conferem resistência à ceftazidima quando produzidas em níveis basais. O aumento da produção destas enzimas confere resistência às cefalosporinas, com exceção do cefepime e carbapenemas (CORVEC et al, 2003).

Centenas de beta-lactamases com características bioquímicas e moleculares distintas têm sido descritas entre os isolados clínicos. Bush, Jacoby e Medeiros desenvolveram um esquema de classificação das beta-lactamases baseado nas características funcionais (BUSH; JACOBY; MEDEIROS, 1995) e incluíram a classificação proposta por Ambler, na qual as beta-lactamases são divididas em 4 classes: **A** (serino beta-lactamases, incluindo as beta-lactamases de espetro ampliado (ESBL), penicilinases e carbenicilinases), **B** (metalo-beta-lactamases), **C** (cefalosporinases cromossomais) e **D** (oxacilinases), baseadas nas sequências de aminoácidos (AMBLER, 1980; ZAVASCKI et al, 2010).

As classes A, C e D têm no sítio ativo uma serina e são inibidas pelo ácido clavulânico. A classe B corresponde às metalo beta-lactamases (MBL) e tem no sítio ativo um íon (zinco), sendo inibida pelos quelantes iônicos, como por exemplo o ácido etileno-diaminotetracético (EDTA) e o ácido 2-mercaptopropiônico (MPA) (DALMARCO; BLATT; CÓRDOVA, 2006).

1.6.1. Beta-lactamases de Espectro Estendido – ESBL

As ESBL estão classificadas dentro do grupo funcional 2, segundo Bush e colaboradores (1995) e da classe A de Ambler (1980) e, atualmente, representam o maior grupo de beta-lactamases estudadas. Estas enzimas são capazes de hidrolisar e causar resistência ou sensibilidade diminuída às penicilinas, oximino-cefalosporinas (cefotaxima, ceftriaxona, ceftazidima, cefepime) e monobactâmicos (aztreonam), mas não às cefamicinas (cefotaxima) e aos carbapenêmicos (imipenema, meropenema, ertapenema); são inibidas por compostos como o ácido clavulânico (clavulanato), o sulbactam e o tazobactam (DHILLON; CLARK, 2012).

São a principal causa da resistência bacteriana aos antibióticos beta-lactâmicos. Elas formam um grande grupo de enzimas capazes de hidrolisar o anel beta-lactâmico de cefalosporinas de terceira e quarta gerações e aztreonam e são inativadas por inibidores específicos, como clavulanato, sulbactam e tazobactam.

Os genes que codificam as ESBL são mais comumente encontrados em membros da família Enterobacteriaceae, porém os elementos genéticos móveis, como plasmídeos, transposons e integrons têm contribuído para o aumento da incidência de genes *bla*$_{\text{ESBL}}$ entre outras bactérias Gram-negativas, incluindo *Pseudomonas aeruginosa*. O isolamento de cepas produtoras de ESBL multirresistentes que levam a um aumento considerável de morbidade por infecções bacterianas, tornou-se o alvo de grandes estudos na área clínica.

A emergência e a disseminação de beta-lactamases de espectro estendido (ESBL) entre os membros da família Enterobacteriaceae têm sido descritas mundialmente como ponto de urgência clínica devido à grande incidência desses microrganismos em infecções relacionadas com a assistência à saúde (IRAS) (SILVA; LINCOPAN, 2012).

A classe A, de Ambler são as penicilinases e cefalosporinases, já as oxa-derivadas são ESBL pertencentes a classe D (oxacilinases), ambas usualmente encontradas em plasmídeos e transposons.

Existem relatos da ocorrência de amostras de *P. aeruginosa* produtoras de ESBL em todos os continentes, evidenciando a grande disseminação desses determinantes de resistência. Várias classes de ESBL (A, B e D) foram encontradas

em *P. aeruginosa*, sendo que cinco tipos de ESBL classe A foram descritas recentemente (PER, VEB, GES, TEM e SHV) (WELDHAGEN; POIREL; NORDMANN, 2003; BAHMANI; RAMAZANZADEH, 2013).

Em 1993, PER-1 foi a primeira ESBL da classe A de Ambler a ser caracterizada em uma amostra de *P. aeruginosa.* Essa enzima foi detectada em amostras provenientes da Turquia. Posteriormente, PER-1 foi encontrada na Síria, Irã, Iraque, Leste Europeu, França, Bélgica e Itália (NORDMANN et al, 1993; CLAEYS et al, 2000; PICÃO; GALES, 2007).

O primeiro relato de produção de ESBL em *P. aeruginosa* no Brasil, data de junho de 2002. A amostra clínica produtora de GES-1 foi isolada de uma paciente submetida a histerectomia por uma neoplasia de endométrio e desenvolveu infecção do sítio cirúrgico no Hospital São Paulo da Universidade Federal de São Paulo. (CASTANHEIRA et al, 2004; PICÃO; GALES, 2007).

Posteriormente, foram detectadas amostras clínicas produtoras de *bla*$_{\text{CTX-M-2}}$, *bla*$_{\text{GES-1}}$ e *bla*$_{\text{GES-5}}$ entre cepas de *P. aeruginosa* produtoras de ESBL em dois estudos distintos realizados em Hospitais de São Paulo, Brasil (PICÃO et al, 2009a; POLOTTO et al, 2012).

Atualmente, cepas produtoras de ESBL são encontradas também em pacientes da comunidade (DHILLON; CLARK, 2012). É importante destacar que foram identificadas Enterobacteriaceae produtoras de ESBL em diversas amostras ambientais, em fezes de indivíduos saudáveis, em alimentos, em fazendas de agropecuária, e em esgotos, o que sugere que a comunidade pode funcionar como reservatório de cepas produtoras destas enzimas, fato que alerta para a possibilidade de sua expansão global (MESA et al, 2006).

1.6.2. *Klebsiella pneumoniae* carbapenemase – KPC

Até o momento já foram descritas diversas carbapenemases. Entre elas destaca-se a KP carbapenemase (KPC). As KPC foram identificadas em praticamente todos os membros de importância clínica da família Enterobacteriaceae e em espécies de não-fermentadores como a *Pseudomonas*

aeruginosa e *Acinetobacter baumannii* (ROBLEDO; AQUINO; VÁZQUEZ, 2011). São classificadas como classe A de Ambler e até o momento, já foram descritas 19 variantes desta enzima (KPC-2 a KPC-20) de acordo com o site www.lahey.org/studies (BUSH; JACOBY, 2015). Tem a capacidade de hidrolisar todos os antibióticos beta-lactâmicos, incluindo as cefalosporinas de primeira, segunda e terceira geração, monobactâmicos e carbapenêmicos, no entanto, são inibidas pelo ácido clavulânico e tazobactam (BUSH; JACOBY, 2010).

Desde o início desta década, enterobactérias que produzem KPC foram relatadas nos EUA e posteriormente em todo o mundo. Estas bactérias produtoras de KPC estão predominantemente envolvidas em infecções nosocomiais e sistêmicas; embora elas estejam presentes, na maior parte, em Enterobacteriaceae, elas também podem ser, raramente, isoladas de *P. aeruginosa* (NORDMANN; CUZON; NAAS, 2009).

As enzimas KPC são encontradas principalmente em *K. pneumoniae*, embora existam relatos cada vez mais crescentes da presença em outros gêneros da família Enterobacteriaceae. Bactérias não fermentadoras como *Pseudomonas* e *Acinetobacter* também têm sido citadas como produtoras de KPC (NORDMANN; CUZON; NAAS, 2009; WALSH, 2010). A rápida disseminação entre diferentes espécies bacterianas Gram-negativas se deve, provavelmente, à localização dos genes *bla*KPC em plasmídeos transmissíveis e a sua associação a transposons (PICÃO et al, 2013).

O primeiro relato de isolados produtores de KPC no Brasil ocorreu em 2009, referente a quatro cepas de *K. pneumoniae* produtoras de KPC-2, isoladas na cidade de Recife em 2006 (MONTEIRO et al, 2009). Peirano e colaboradores (2009) caracterizaram 6 cepas produtoras de KPC-2 nos anos de 2007 e 2008 no Rio de Janeiro.

Jácome e colaboradores (2012) descreveram a primeira detecção de um isolado de *P. aeruginosa* produtora de KPC-2 no Brasil. Esses isolados foram provenientes de secreção traqueal de dois pacientes distintos internados em uma unidade de tratamento intensivo de um hospital localizado em Pernambuco.

Os isolados produtores de KPC estão ocorrendo em muitas regiões brasileiras. Um estudo realizado entre agosto de 2009 e outubro de 2010, revelou os seguintes casos: Alagoas (1), Distrito Federal (207), Minas Gerais (38),

Pernambuco (38), Rio de Janeiro (42), São Paulo (70), Tocantins (1), Goiás (4), Espírito Santo (6), Paraíba (18), Paraná (24); Bahia e Ceará, respectivamente, 2 e 150 casos suspeitos (JASKULSKI, 2013).

Desde a descrição inicial de KPC no Brasil, várias publicações têm demonstrado a sua disseminação em todo o país, e sua presença em diversos gêneros e espécies. A disseminação de enterobactérias produtoras de KPC é um grave problema clínico e epidemiológico em diversas instituições de saúde brasileiras (MONTEIRO et al, 2009). Atualmente, KPC constitui importante mecanismo de resistência no contexto hospitalar mundial. Sua pesquisa é relevante a fim de limitar sua disseminação, contribuindo para a redução dos índices de morbidade e mortalidade ligados a diferentes doenças infecciosas, em que é imprescindível a vigilância microbiológica, juntamente com ação da Comissão de Controle de Infecção Hospitalar (CCIH) (DIENSTMANN et al, 2010).

1.6.3. Metalo-Beta-Lactamases – MBL

As MBL têm um amplo espectro de atividade hidrolítica contraem beta-lactâmicos, incluindo penicilinas, cefalosporinas e carbapenêmicos, mas não aos monobactâmicos. As MBL são beta-lactamases da classe B de Ambler e podem ser produzidas intrinsicamente por alguns microrganismos, ou podem ser adquiridas. A produção constitutiva dessas enzimas é carreada no cromossomo.

Estas enzimas foram denominadas de MBL móveis ou adquiridas, devido ao fato dos genes serem inseridos em estruturas genéticas que fornecem mobilidade. Assim, as MBL adquiridas são codificadas por cassetes gênicos localizados no cromossomo ou plasmídeos bacterianos, com exceção da enzima SPM-1, que é codificada por um gene localizado em plasmídeo. As demais são codificadas por genes localizados em integrons de classe 1 (MENDES et al, 2006).

Atualmente são conhecidas nove subclasses de MBL: IMP, VIM, SPM, GIM, SIM, AIM, KHM, NDM e DIM. Essas MBL têm se tornado clinicamente importantes, principalmente, em *Pseudomonas* spp., *Acinetobacter* spp., e alguns gêneros da família Enterobacteriaceae.

A enzima NDM-1 é uma das mais recentes MBL adquiridas e sua propensão à disseminação causa grande preocupação. Esta enzima foi identificada pela primeira vez em 2008 e desde então tem sido amplamente descrita em enterobactérias causando infecções esporádicas e surtos, principalmente no subcontinente indiano (JOHNSON; WOODFORD, 2013). Poucos casos de *K. pneumoniae* produtoras de NDM foram descritos na América Latina (PASTERAN et al, 2012) e até o momento esta carbapenemase não tinha sido detectada em nosso país. Recentemente foram detectados casos de microrganismos produtores de NDM-1 no estado do Rio Grande do Sul, na cidade de Porto Alegre. O gene $bla_{\text{NDM-1}}$ foi identificado em *Providencia rettgeri* e *Enterobacter cloacae* (BRASIL, 2013).

A primeira MBL caracterizada em *P. aeruginosa* denominada VIM-1, foi descrita em Verona, Itália (LAURETTI et al, 1999). A enzima IMP parece ser mais prevalente na Ásia, sendo comumente encontrada em bacilos Gram-negativos não-fermentadores (BGN-NF) (*Achromobacter xylosoxidans*, *Pseudomonas* spp. e *Acinectobacter* spp.) (MENDES et al, 2006). Variantes de VIM e IMP têm sido relatados em todo o mundo, entre *Pseudomonas* spp., *Acinetobacter* spp. ou Enterobacteriaceae. Até o momento 25 variantes de IMP e 23 de VIM já foram descritas (ZAVASCKI et al, 2010).

Os tipos de MBL detectadas no mundo, incluindo o Brasil são SPM, IMP e VIM (WALSH, 2010; CORNAGLIA; GIAMARELLOU; ROSSOLINI, 2011). As do tipo SPM são as mais frequentemente encontradas no Brasil e têm sido detectadas em São Paulo (SP), Salvador (BA), Curitiba, Londrina e Maringá (PR), Brasília (DF), Santo André (SP) e Fortaleza (CE) (GALES et al, 2003). As IMP foram detectadas nas cidades de Brasília (MENDES et al, 2004), São Paulo (SADER et al, 2005), Rio de Janeiro (CARVALHO et al, 2006) e Porto Alegre (ZAVASCKI et al, 2007a) e as VIM, em São Paulo (SADER et al, 2005).

1.7. EFLUENTE HOSPITALAR

Fora de um ambiente hospitalar, as pressões que o ambiente exerce são de outra natureza, tais como: temperatura, pH, concentração de nutrientes,

concentração de metais pesados e as próprias interações populacionais, entre outras (BANNING; TOZE; MEE; 2003).

Os efluentes hospitalares, devido à presença conjunta de microrganismos patogênicos e compostos químicos como desinfetantes, anestésicos, metais pesados, antibióticos e outras drogas não metabolizadas por pacientes (EMMANUEL et al, 2005), representam grande risco para a saúde pública. Cerca de 30 a 60% dos medicamentos prescritos pela clínica médica são excretados no efluente hospitalar (McQUILLIAN et al, 2002). Al-Ahmad e colaboradores (1999) verificaram que muitos antibióticos não são metabolizados completamente após o seu lançamento em sistemas de coleta e tratamento de esgotos, permanecendo ativos na fase líquida por mais de 20 dias, exercendo ação seletiva sobre a comunidade bacteriana presente. A disposição desse resíduo líquido nas estações de tratamento de esgoto hospitalar pode desencadear, pela pressão seletiva, um aumento de populações bacterianas com fenótipos de multirresistência aos antimicrobianos (PRADO et al, 2008).

A Comissão Parlamentar de Inquérito da Saúde da Câmara de Vereadores da Cidade do Rio de Janeiro constatou que o esgoto produzido por diversos hospitais da cidade é despejado no mar, tanto na Baía de Guanabara como pelo emissário de Ipanema. De acordo com o estudo, pelo menos 101 dos 420 hospitais existentes na cidade não têm estação de tratamento e, destes, 69 despejam seus resíduos líquidos no mar. Vinte e quatro hospitais encaminham seu esgoto para estações da Companhia Estadual de Águas e esgotos (CEDAE), de onde ele é descartado pelo emissário de Ipanema.

Pesquisadores afirmam que o tratamento dado ao esgoto não é suficiente para matar bactérias, vírus e parasitas, o que simplesmente colocaria sob risco as praias do Rio de Janeiro (Ipanema, Leblon e Arpoador), que poderiam estar contaminadas pelo esgoto despejado por 28 hospitais. Na Baía de Guanabara, a situação constatada é ainda pior, pois ela recebe o esgoto de 41 hospitais sem nenhum tratamento. Já o complexo lagunar de Jacarepaguá (lagoas de Marapendi, Camorim, Jacarepaguá e da Tijuca) recebe o esgoto de oito hospitais. Esta carga poluente acaba chegando à praia de Barra da Tijuca através do Quebra-Mar, e isso significa que o banho ali é de altíssimo risco. O esgoto hospitalar tem uma concentração de bactérias e vírus três vezes maior que a do esgoto domiciliar, e

assim os riscos de contaminação por contato são muito maiores. Há um consenso entre os pesquisadores de que todos os hospitais deveriam possuir Estações de Tratamento de esgoto adequadas (ABESSA, 2002).

No Brasil, a grande preocupação ainda está centralizada nos danos ambientais e à saúde causados por macropoluentes, uma vez que, de acordo com o Sistema Nacional de Informações sobre Saneamento (SNIS) (BRASIL, 2011), de todo o esgoto gerado no país, apenas 37,9% recebe algum tipo de tratamento.

A presença de bactérias multirresistentes em efluentes hospitalares também foi verificada em uma estação de tratamento de esgoto hospitalar na cidade do Rio de Janeiro, onde pesquisadores isolaram uma cepa de *K. pneumoniae* produtora de ESBL (beta-lactamase de espectro estendido) e concluíram que a estação de tratamento de esgoto analisada não foi capaz de reduzir eficientemente a alta concentração de coliformes totais e fecais (PRADO et al, 2008). Outro estudo realizado no sul do Brasil concluiu que 60% das cepas de *Acinetobacter* spp. isoladas de um efluente hospitalar eram multirresistentes aos antimicrobianos testados e metade das cepas produziam ESBL (GUSATTI et al, 2009).

P. aeruginosa é um dos principais microrganismos recuperados de efluentes hospitalares (FUENTEFRIA et al, 2008) e os genes de resistência aos carbapenêmicos bla_{VIM-2} e $bla_{SPM\ -1}$, bastante disseminados entre cepas de *P. aeruginosa* de origem clínica, já foram detectados em isolados ambientais aquáticos no Brasil e em Portugal, respectivamente (FUENTEFRIA; FERREIRA; CORÇÃO, 2011). Assim, os efluentes hospitalares, quando não são tratados apropriadamente, contribuem para a disseminação de bactérias hospitalares multirresistentes aos antimicrobianos para o ambiente extra-hospitalar como lagos, rios e oceanos comprometendo a saúde da população (YANG et al, 2009).

1.8. ESTAÇÃO DE TRATAMENTO DE ESGOTO HOSPITALAR

Os processos utilizados para tratamento de efluentes, de modo geral, devem levar em conta as características do efluente a ser tratado, bem como o grau de recepção do corpo d’água onde o mesmo será lançado.

O gerenciamento e tratamento de efluentes hospitalares variam muito em diferentes países. Tanto em países desenvolvidos como àqueles em desenvolvimento podem estar completamente ausentes, o que significa que o efluente hospitalar é descarregado diretamente na superfície de corpos d'água; ou submetidos à simples cloração; ou à clarificação primária seguida pela cloração; ou tratamentos primários e secundários seguidos de desinfecção química (PRAYITNO et al, 2014). Vários projetos de pesquisa têm sido realizados nesses países, com o objetivo de avaliar a adequação de tratamentos para efluente hospitalar. Eles referem-se geralmente a uma discussão da eficiência de remoção de contaminantes convencionais e microrganismos, e das possibilidades de reutilizar esta água recuperada para irrigação considerando problemas decorrentes da escassez de água (SHRESTHA; HABERL; LABER, 2001; CHITNIS et al, 2004; BEYENE; REDAIE, 2011; ABDEL-GAWAD; ALY, 2011).

Esses tratamentos podem ser classificados em função do tipo de impurezas retiradas e/ou do seu grau de remoção.No caso dos efluentes hospitalares, basicamente, as estações de tratamento são estruturadas da seguinte forma: uma etapa preliminar, em que o efluente hospitalar chega à estação de tratamento e ocorre a remoção de grandes sólidos e areia através de grades instaladas na entrada da estação; a etapa seguinte é a fase de tratamento primário ou físico químico, em que os sólidos em suspensão não grosseiros são removidos por meios físicos, através de unidades de sedimentação, para que a matéria orgânica contida no efluente seja reduzida.

Na fase de tratamento primário ou físico químico, o afluente chega à estação de tratamento e são direcionados a um poço situado abaixo do nível da edificação, em que os sólidos em suspensão não grosseiros são removidos por meios físicos, através de unidades de sedimentação, para que a matéria orgânica contida no efluente seja reduzida para que sejam protegidas as demais unidades de tratamento, além dos dispositivos de transporte (bombas e tubulações) e os corpos receptores. Neste poço, o afluente percorre por calhas (abertas) e por uma em especial, calha Parshall, onde a vazão do líquido é medida por períodos pré-determinados. Com o auxílio de bombas de recalque, a massa líquida é transferida para o tanque de aeração, onde é injetado, por um período pré-determinado, oxigênio em grande quantidade para que seja estimulada a multiplicação da

microbiota bacteriana aeróbica, iniciando-se a fase de tratamento secundário ou biológico.

Nesta fase são reproduzidos os fenômenos naturais de estabilização da matéria orgânica que ocorrem no corpo receptor, sendo que a diferença está na maior velocidade do processo, na necessidade de utilização de uma área menor e na evolução do tratamento em condições controladas. A base de todo o processo biológico é o contato efetivo entre os microrganismos aeróbios e o material orgânico contido nos efluentes, de tal forma que esse possa ser utilizado como alimento pelos microrganismos. Eles convertem a matéria orgânica em gás carbônico, água e material celular.

Após a fase em que é feita a degradação biológica, os sólidos produzidos são depositados no fundo do tanque formando o lodo (lodo primário). A parte líquida é dirigida ao tanque de decantação e o sobrenadante flui vagarosamente pelos decantadores para o tanque de esgoto tratado. No fundo do tanque de decantação também há formação de um lodo que juntamente como o lodo primário, retorna ao tanque de aeração para mais uma vez refazer o ciclo e ter melhor rendimento do efluente. O retorno do lodo é necessário para suprir o tanque de aeração com uma quantidade suficiente de microrganismos e manter uma relação alimento/microrganismo capaz de decompor com maior eficiência o material orgânico (SANTORO, 2011).

O esgoto tratado é então encaminhado a um tanque de passagem de dimensões menores que os anteriores, localizado na área externa à estação onde é adicionado, por gotejamento, cloro líquido que é a fase terciária de tratamento. A quantidade do cloro é calculada de acordo com a vazão de saída do efluente e provém de uma “bomba dosadora de cloro”, instalada no núcleo central de operações da estação de tratamento. O efluente clorado é então incorporado à rede pluvial. O lodo resultante de todo o tratamento é estocado e retirado periodicamente por caminhões tanque, e encaminhado para a estação de tratamento de esgoto Alegria, Rio de Janeiro (NOVAES, 2008).

Países como EUA, Nova Zelândia e Austrália têm investido nas chamadas “ilhas flutuantes” (sigla FTWs, em inglês, Floating Treatment Wetlands), que trazem a água suja de volta ao seu estado puro e reutilizável. Cientistas têm buscado uma maneira de limpar lagoas de água pluviais, águas residuais de efluentes e água de

escoamento agrícola, e estudam como as ilhas flutuantes podem purificar essas águas (TANNER; HEADLEY, 2011).

Uma abordagem interessante foi adotada na França para gerir e tratar o efluente do Centre Hospitalier Alpes Léman em Annemasse. Por meio de uma tubulação o efluente hospitalar é transportado para um local próximo da Estação de tratamento de esgoto doméstico, onde ele é tratado numa linha específica e submetido a contínua monitorização para melhorar a remoção de compostos persistentes. Esta foi uma decisão tomada pelas autoridades locais, que ainda elaboraram uma legislação específica para este propósito (RELATÓRIO SIPIBEL, 2014).

1.9. TIPIFICAÇÃO MOLECULAR DA *Pseudomonas aeruginosa*

As técnicas moleculares de tipificação podem ser utilizadas para investigação epidemiológica de um surto infeccioso e para determinar a origem e a disseminação de diversos clones.

A aplicação e a interpretação de ferramentas de tipificação microbiana em estudos epidemiológicos requerem a compreensão das limitações das técnicas. Além da confiabilidade, uma técnica é considerada válida quando sua habilidade de discriminação entre cepas é satisfatória e uma base biológica para o agrupamento de cepas com tipos aparentemente distintos é possível (FOXMAN et al, 2005).

Tipificações baseadas na reação em cadeia da polimerase (PCR) são técnicas moleculares rápidas e baratas, como as técnicas: *Enterobacterial Repetitive Intergenic Consensus* - PCR (ERIC-PCR) e *Random Amplified Polymorphic DNA* - PCR (RAPD-PCR) (WILSON; SHARP, 2006) e podem ser utilizadas na análise da diversidade genética de diferentes microrganismos tanto no nível inter e intraespecífico e tem servido como protocolo preliminar para as técnicas potencialmente mais discriminatórias e elaboradas como a Eletroforese em gel de Campo Pulsado (PFGE) e *Multilocus Sequence Typing* (MLST).

1.9.1. *Enterobacterial Repetitive Intergenic Consensus* – ERIC

A família de elementos repetitivos chamada *Enterobacterial Repetitive Intergenic Consensus* (ERIC) é um palíndromo imperfeito de 127 pb que ocorre em múltiplas cópias no genoma de bactérias entéricas e víbrios e podem servir como iniciadores para a amplificação do DNA genômico (VERSALOVIC; KOEUTH; LUPSKI, 1991). Essas regiões localizadas entre os elementos ERIC variam de tamanho entre as cepas, gerando perfis de bandas únicos e característicos quando separados por eletroforese em géis de agarose (COSTA et al, 2006).

Versalovic e colaboradores (1991) propuseram essa metodologia para tipificação de genomas bacterianos, inclusive para *Pseudomonas aeruginosa*. Essa metodologia tem se mostrado com alto poder discriminatório e de reprodutibilidade (SILBERT et al, 2004).

A utilização da técnica de ERIC-PCR para tipificação de *Pseudomonas aeruginosa* já é bem estabelecida e estudos têm demonstrado que essa metodologia é rápida e eficiente, além da alta reprodutibilidade, na discriminação das linhagens desta espécie (INACIO et al, 2014) o que permite o rastreamento de cepas epidêmicas durante surtos (De ABREU et al, 2014).

1.9.2. *Multilocus Sequence Typing* – MLST

Estudos de genética de populações requerem amostragens estruturadas não só das características fenotípicas, mas também do genoma dos organismos estudados. A comparação dos genomas bacterianos utilizada nesses estudos pode envolver o genoma inteiro ou amostras representativas do mesmo podendo conter ou não referências às sequências genômicas anotadas. A comparação direta do DNA genômico de dois ou mais isolados pode ser realizada de várias formas como, por exemplo, a identificação de grupos de genes presentes em um isolado e ausentes em outro. Outra alternativa é a análise da variação de grupos de genes em múltiplos isolados como por exemplo no método do MLST.

O MLST desenvolvido por Maiden e colaboradores (1998) é um método de tipificação baseado no sequenciamento do DNA genômico, que permite a detecção de variações em genes constitutivos chamados “housekeeping genes” utilizados preliminarmente para a identificação de grupos (“clusters”) de isolados com genótipos idênticos ou altamente relacionados, chamados de clones ou complexos clonais.

O MLST apresenta as seguintes vantagens para os estudos de genética de populações: (a) a caracterização de múltiplos loci produz uma variedade de dados consistentes permitindo análises mais robustas; (b) as sequências de nucleotídeos geradas permitem análise inter-laboratorial rápida podendo ser facilmente analisadas por inúmeros programas para estudos filogenéticos e de genética de populações; (c) os genes constitutivos fornecem alta discriminação para a tipificação bacteriana e apresentam evolução lenta por não estarem sujeitos a pressões seletivas que poderiam obscurecer possíveis relações genéticas entre diferentes isolados; (d) os dados gerados são acessíveis pela internet, através de bancos de dados virtuais para comparação dos perfis genéticos permitindo o monitoramento da circulação de clones epidêmicos em todo o mundo.

Este método foi desenvolvido para reduzir a dificuldade de comparação de resultados entre laboratórios por parte das técnicas moleculares tradicionais (SABAT et al, 2013). Curran e colaboradores (2004) estabeleceram as condições para o estudo do MLST para a tipificação molecular dos isolados de *P. aeruginosa* que deu origem a um banco de dados. Atualmente, esse banco contém 3179 depósitos distribuídos em 2226 ST e apenas 2 Complexos Clonais (PA01 e PA14) com isolados de *P. aeruginosa* de espécimes clínicos, de solo, de água doce e efluente hospitalar.

No Brasil, até o momento, foram depositadas 149 cepas, isoladas entre 1999 e 2015, o que corresponde a 4,7% do total de cepas do banco de dados. A maioria (83%) dos isolados foi proveniente da região Sudeste do Brasil: 14 (9%) de Belo Horizonte, 16 (11%) de São Paulo e 94 (63%) do Rio de Janeiro. Dessas cepas, 81 (54%) foram de isolados clínicos (sangue, infecção do trato urinário, infecção de tecidomole e secreção), 20 (13%) do efluente hospitalar, 2 (2%) da água e 46 (31%) de outras fontes não divulgadas.

2. RELEVÂNCIA

Estudos na área da Saúde Ambiental apresentam atualmente um campo ainda pouco explorado. Neste projeto, a prioridade dada aos poluentes microbiológicos é justificada pelos possíveis impactos negativos a serem gerados devido ao lançamento de efluentes hospitalares, contendo microrganismos e genes de resistência aos antimicrobianos, nos corpos hídricos.

A ANVISA e o CONAMA têm assumido o papel de orientar, definir regras e regular a conduta dos diferentes agentes, no que se refere à geração e ao manejo dos resíduos de serviços de saúde, com o objetivo de preservar a saúde e o meio ambiente, garantindo a sua sustentabilidade. Além disso, no Rio de Janeiro, existe um programa de controle, administrado pela Subsecretaria de Águas Municipais (Rio Águas) e a Secretaria Municipal de Saúde, criado para atender a Lei municipal nº 2661/96, que regulamenta o disposto no art. 277 da Constituição do Estado do Rio de Janeiro, no que se refere à exigência de níveis mínimos de tratamento de esgotos hospitalares sanitários, antes de seu lançamento em corpos d'água.

Apesar de todas essas regulamentações, um estudo realizado pela Secretaria Municipal de Meio Ambiente (SMAC) demonstrou que existem 420 unidades de saúde em toda a cidade do Rio de Janeiro e destas unidades, 361 foram vistoriadas e 197 tiveram seu efluente analisado. O resultado encontrado revelou que 75% não possuem estação de tratamento de esgoto e dentre elas 56% também não apresentam nenhum tratamento final no destino de seu efluente. Este estudo ainda verificou que 40 unidades de saúde têm seu efluente contaminado por *P. aeruginosa*, oriundo de fezes e sangue de pacientes contaminados (DIÁRIO OFICIAL DO MUNICÍPIO DO RIO DE JANEIRO, 2002).

Os dados da Comissão Parlamentar de Inquérito (CPI) da Saúde da Câmara de Vereadores da cidade do Rio de Janeiro constataram que o esgoto produzido por diversos hospitais da cidade é despejado no mar, tanto na Baía de Guanabara como pelo emissário de Ipanema.

A realização deste estudo é fundamentada na necessidade de atender à legislação vigente no Brasil, em relação ao descarte de resíduos líquidos dos Serviços de Saúde e no estabelecimento da relação entre a Vigilância Ambiental e a

Saúde Pública. As metodologias para estudos que dizem respeito à relação da saúde com o ambiente, são necessariamente mais diversas e complexas do que nas outras áreas da Saúde, uma vez que, a diversidade do conceito de ambiente amplia o número de questões/objetos de interesse, que exigem diferentes formas de abordagem metodológica (CÂMARA; TAMBELLINI, 2003).

Nós optamos por uma abordagem polifásica capaz de estabelecer o perfil clonal dos isolados de *P. aeruginosa* hospitalares e do efluente do mesmo hospital associando aos perfis de susceptibilidade aos antibióticos, focando em particular aos mecanismos baseados em enzimas de resistência aos beta-lactâmicos e estabelecer as relações filogenéticas entre os isolados.

Até o momento, não temos conhecimento de nenhum estudo utilizando essa abordagem em isolados do hospital e de seu efluente simultaneamente, com ênfase na microbiologia clínica e aspectos de impacto ambiental. Os resultados obtidos poderão revelar dados inéditos no Brasil. Com base nessas evidências nós acreditamos que a realização do mesmo poderá nos permitir uma melhor avaliação da influência do tratamento no comportamento de patógenos multirresistentes. Desta forma, contribuir para o aprimoramento das ações da vigilância ambiental e epidemiológica o que caracteriza sua extrema relevância para a Saúde pública.

3. OBJETIVOS

3.1. GERAL

Estabelecer o perfil clonal entre os isolados de *Pseudomonas aeruginosa* de origem clínica e do efluente hospitalar, associados à susceptibilidade frente aos antibióticos e suas relações filogenéticas.

3.2. ESPECÍFICOS

- Identificar as cepas de *P. aeruginosa* isoladas de infecções clínicas e do efluente hospitalar por metodologia fenotípica e molecular;
- Avaliar a susceptibilidade aos antimicrobianos dos isolados e estabelecer os perfis de resistência;
- Detectar fenotipicamente a presença de ESBL nos isolados que forem resistentes à cefepima e ceftazidima;
- Identificar fenotipicamente a atividade carbapenemase nos isolados que forem resistentes ao imipenema;
- Investigar a presença de genes que codificam as ESBL, MBL e KPC;
- Determinar o perfil clonal dos isolados de *P. aeruginosa* hospitalares e do efluente hospitalar e correlacionar com a susceptibilidade aos antimicrobianos;
- De acordo com os resultados obtidos, prever os impactos negativos e os agravos ao ambiente e à saúde pública.

4. METODOLOGIA

4.1. LOCAL DO ESTUDO

4.1.1. Complexo Hospitalar: Hospital Municipal Lourenço Jorge – Maternidade Leila Diniz (HMLJ)

O Hospital Municipal Lourenço Jorge tem perfil de grande emergência, possui 258 leitos, distribuídos entre os setores de cirurgia geral, bucomaxilofacial e ortopedia, traumatologia; clínica geral, neonatologia e de AIDS, UTI adulto, 12 unidades intermediárias neonatais, unidade de isolamento, unidade intermediária, UTI neonatal, obstetrícia clínica e cirúrgica, pediatria clínica, tisiologia e psiquiatria. A Maternidade Leila Diniz, que por sua vez, possui 105 leitos distribuídos nos setores de cirurgia geral e ginecológica, clínica geral e neonatologia, UTI neonatal, unidade intermediária neonatal, obstetrícia clínica e cirúrgica. As duas unidades, localizadas na Cidade do Rio de Janeiro, totalizam 363 leitos e realizam cerca de 30 mil atendimentos mensais. (Cadastro Nacional Estabelecimentos de Saúde – CNES Net-MS, 2004).

4.1.2. Estação de Tratamento de Esgoto Hospitalar (ETEH)

A Estação de Tratamento de Esgoto do complexo Lourenço Jorge-Leila Diniz tem a capacidade para tratar 220 m^3 de esgoto por dia, evitando que o esgoto produzido pelo hospital seja despejado sem tratamento nos rios da região. O tratamento do esgoto é classificado como terciário, ou seja, além de realizar a fase primária e secundária, completa o tratamento com uma fase terciária que, no caso específico desta estação, se trata da adição de um processo químico.

4.2. AMOSTRAGEM

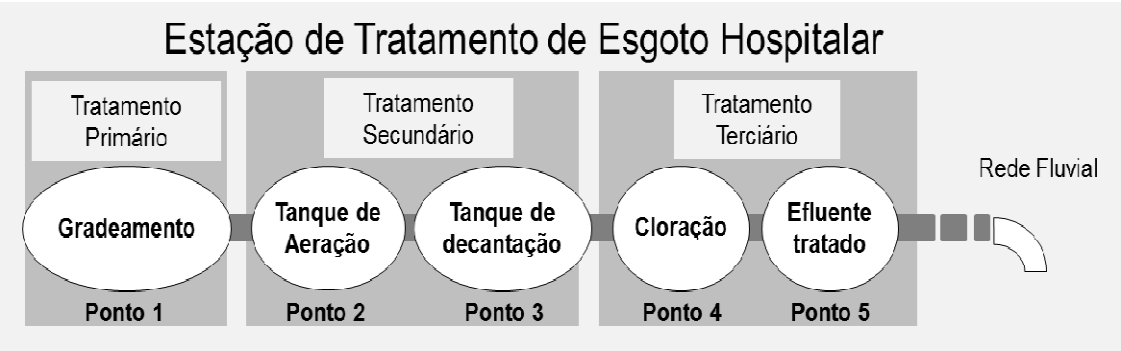
4.2.1. Coleta e Identificação de Amostras Clínicas

As amostras clínicas foram provenientes de infecções relacionadas à assistência à saúde, coletadas no período de 2008 a 2012, identificadas como *P. aeruginosa* pelo sistema Vitek II do Laboratório de Patologia Clínica do Hospital Municipal Lourenço Jorge.

4.2.2. Coleta e Identificação das Amostras provenientes da Estação de Tratamento de Esgoto Hospitalar (ETEH)

Amostras de 500 mL foram coletadas assepticamente, em triplicata com intervalos de 15 minutos em garrafas esterilizadas, em 2008 e 2010, de cada uma das cinco etapas da estação de tratamento estudados: Gradeamento/afluente da ETEH (ponto 1), tanque de aeração (ponto 2), tanque de decantação (ponto 3), Cloração (ponto 4) e efluente tratado (ponto 5) (Esquema 1). Em cada ponto foi realizada a dosagem de pH, turbidez, condutividade, oxigênio dissolvido, temperatura, salinidade com o equipamento Water Quality Checker U-10 (HORIBA) e a dosagem de cloro com o medidor de cloro (Homis). As amostras foram colocadas no gelo (4ºC) até serem processadas no laboratório. A amostra foi encaminhada para o Setor de Bactérias de Referência onde realizamos o isolamento e identificação de *P. aeruginosa*.

Esquema 1. Pontos de coleta da ETEH



Os 500 mL, de cada ponto estudado da ETEH, foram filtrados em membranas de porosidade de 0,45 µm. As membranas foram transferidas para o caldo de asparagina selectivo, incubados a 37ºC durante 24 h. O aparecimento de fluorescência verde sob luz ultravioleta foi considerado como resultado positivo, indicativo da presença de bactéria do gênero *Pseudomonas*. Alíquotas de 1 mL, a partir destes tubos positivos, foram transferidos para caldo acetamida e incubadas a 37°C durante 24 h. Aparecimento da cor rosa no tubo foi considerado positivo, e semeadas em agar acetamida, em duplicata para cada ponto (FUENTEFRIA; FERREIRA; CORÇÃO, 2011). Após incubação, foram selecionadas 6 colônias de cada placa. Coloração de Gram e provas bioquímicas convencionais, como citocromo-oxidase, fermentação e oxidação da glicose, fermentação e oxidação da lactose, crescimento em caldo simples a 42°C, motilidade, produção de H_2S, redução de nitrato e produção de pioverdina foram utilizadas, de acordo com o Manual Bergey (BERGEY'S..., 2011), para identificar as cepas.

4.3. CERTIFICAÇÃO MOLECULAR DOS ISOLADOS

Todos os isolados (clínicos e do efluente hospitalar), previamente identificados fenotipicamente, foram certificados por metodologia molecular. A extração do DNA genômico foi realizada utilizando o protocolo para Gram negativo do kit Dnaeasy®Blood&Tissuet (Qiagen®) de acordo com as instruções do fabricante. Foi realizada a PCR do gene 16S rRNA com iniciadores específicos para

o gênero: PA-GS-F (5’-GACGGGTGAGTAATGCCTA-3’) e PA-GS-R (5’-CACTGGTGTTCCTTCCTATA-3’) e espécie: PA-SS-F (5’-GGGATCTTCGACCTCA-3') e PA-SS-R (5’-TCCTTAGAGTGCCACCCG-3’) descritos por Spilker e colaboradores (2004). O DNA genômico foi analisado em gel de eletroforese por 1 hora a 50 V em gel de agarose 1% no tampão TBE 0.5X e corado com brometo de etídio (3mg/mL). O gel foi fotografado e analisado utilizando-se o digitalizador de imagens ImageQuant 300 (GE).

4.4. PRESERVAÇÃO DOS ISOLADOS

Após a identificação fenotípica e certificação molecular, todos os isolados de *P. aeruginosa* (clínicos e do efluente hospitalar) foram preservados por liofilização e depositados na Coleção de Microrganismos de Pesquisa do INCQS/FIOCRUZ com a seguinte identificação: isolados clínicos (P3409 - P3411, P3444 - P3450, P3498 - P3505, P3507 - P3509, P3513 - P3519, P3521 - P3524, P3549 - P3552, P3570 - P3575, P3578 - P3582, P3592 - P3599, P3611 - P3618, P3625 - P3630, P3661 - P3666, P3718 - P3727, P3751 - P3760, P3851, P3852, P3865 - P3868, P3871, P3876, P3877, P3888 - P3890, P3892 - P3894, P3907, P3923 - P3925, P3928, P3929, P3945 - P3949, P3995, P4011, P4014 - P4021, P4034 - P4038); isolados do efluente hospitalar (P3229, P3233 - P3234, P3238 - P3242, P3246, P3249, P3252, P3671 - P3689, P3691 - P3697, P3846 - P3849). A liofilização foi realizada segundo o POP 65.3230.001.

4.5. TESTES DE SUSCEPTIBILIDADE AOS ANTIMICROBIANOS

4.5.1. Método de Disco Difusão (Kirby-Bauer)

A susceptibilidade aos antimicrobianos foi determinada pela técnica de disco-difusão (método de Kirby-Bauer) e os resultados foram interpretados segundo os critérios estabelecidos pelo Clinical Laboratory Standards Institute (CLSI, 2014). As cepas de referência de *Escherichia coli* (ATCC 35218) e *P. aeruginosa* (ATCC 27853) foram utilizadas como controle da qualidade dos discos.

Foram utilizados 17 antibióticos: amicacina (30 µg), aztreonam (30 µg), cefepima (30 µg), cefotaxima (30 µg), ceftazidima (30 µg), ceftriaxona (30 µg), ciprofloxacina (5 µg) gentamicina (10 µg), imipenema (10 µg), meropenema (10 µg), levofloxacina (5µg), (piperacillina/tazobactam (100/10 µg), ticarcillina/ácido clavulânico (75/10 µg), tobramicina (10 µg), polimixina B (300 units), colistina (10 µg) e fosfomicina (200 µg).

Os isolados que apresentaram resistência à ceftazidima e cefepima foram classificados como possíveis produtores de ESBL, aqueles resistentes à imipenema ou meropenema como KPC e à ceftazidima e imipenema como MBL. Os perfis de multidroga resistência (MDR) e extensivamente droga resistente (XDR) foram definidos de acordo com MAGIORAKOS e colaboradores (2012). Valores de $p<0,05$ indicativos de evidência estatística significativamente de recombinação entre o perfil de resistência, de cada ponto da ETEH ou dos locais de isolamento do HMLJ, foram inferidos através do teste de Fisher ou teste Qui-quadrado.

4.5.2. Detecção de *P. aeruginosa* produtoras de ESBL

Foi utilizado o meio Chromoagar – ESBL (CHROMagar, Paris, France) para a detecção de isolados de *Pseudomonas aeruginosa* produtoras de ESBL. A presença de crescimento com coloração translúcida (pigmento natural a verde) indicou o

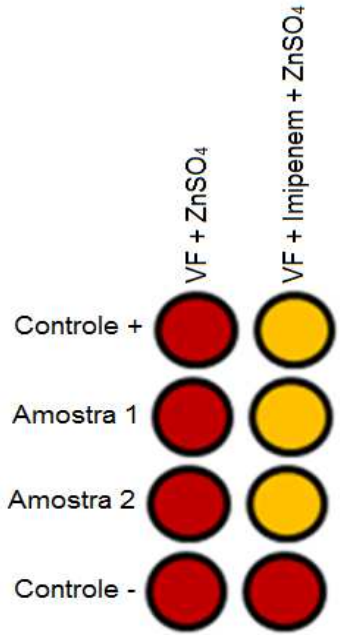
resultado positivo. Este teste foi realizado em amostras resistentes à ceftazidima ou cefepima pela técnica de disco-difusão.

4.5.3. Hidrólise do anel beta-lactâmico - Teste de Carba NP

O teste de Carba NP, baseia-se na detecção bioquímica da hidrólise do anel beta-lactâmico de um antibiótico carbapenêmico, imipenema ou meropenema, seguido da alteração de cor do indicador de pH (vermelho para amarelo) (Nordmann; Poirel; Dortet, 2012).

Este teste foi realizado em amostras resistentes ao imipenema, pela técnica de disco-difusão, cultivadas em placas de agar Mueller-Hinton acrescido de 1 µg/mL de imipenema com cilastatina a 37ºC por 24h. Após o crescimento, foi feita uma suspensão com uma alça de 10 µL repleta de crescimento em 500 µL de Tris-HCl (20mM – pH 7,5). A suspensão foi homogeneizada e em equipamento tipo vortex e a seguir foi realizada sonicação em banho de água (47 kHz. ± 6% / 60 W) por 30 minutos. Foram inoculados 30 µL de cada suspensão em 2 tubos contendo: (1) 100 µL de Vermelho de fenol (VF) + $ZnSO_4$ e (2) 100 µL de vermelho de fenol + $ZnSO_4$ + 3 mg/mL de imipenema. Incubamos a 37°C por até 2 h protegida da luz. A presença da cor alterada (amarelo), representou resultado positivo para a atividade carbapenemase dos isolados (Esquema 2).

Esquema 2. Representação do teste de Carba NP



Resultado positivo: alteração da cor vermelha para amarela; resultado negativo: a cor vermelha permanece inalterada.

4.6. DETECÇÃO DE GENES DE RESISTÊNCIA

Todos os isolados que apresentaram resultado positivo nos testes fenotípicos para possíveis produtores de ESBL, KPC e MBL, foram submetidos à pesquisa dos genes de resistência através da PCR. Foram pesquisados genes codificadores de ESBL (*bla*$_{PER}$, *bla*$_{VEB}$, *bla*$_{SHV}$, *bla*$_{CTX\text{-}M\text{-}1}$, *bla*$_{CTX\text{-}M\text{-}2}$, *bla*$_{CTX\text{-}M\text{-}8}$, *bla*$_{CTX\text{-}M\text{-}9}$, *bla*$_{CTX\text{-}M\text{-}25}$, *bla*$_{TEM}$ e *bla*$_{GES}$), MBL (*bla*$_{IMP}$, *bla*$_{VIM}$, *bla*$_{SPM}$ e *bla*$_{NDM}$) e KPC (*bla*$_{KPC}$).

Os iniciadores e as condições da PCR utilizados, foram previamente descritos (TOLEMAN et al, 2002; DUBOIS et al, 2005; POIREL et al, 2011; MONTEIRO et al, 2012; DOYLE et al, 2012; WANG et al, 2012; BARGUIGA et al, 2013; AHMED et al, 2013) (Tabela 1).

Tabela 1. Sequência dos iniciadores utilizados para a pesquisa dos genes de resistência

Gene	Iniciadores	Sequência	Amplicon (pb)	Referência
bla$_{PER}$	per+	5' - CCTGACGATCTGGAACCTTT – 3'	716	Barguiga, 2013
	per-	5' – GCAACCTGCGCAAT(GA)ATAGC – 3'		
bla$_{VEB}$	VEB+	5' – ATTTCCCGATGCAAAGCGT – 3'	542	Barguiga, 2013
	VEB –	5' – TTATTCCGGAAGTCCCTGT – 3'		
bla$_{SHV}$	SHV-F	5' – ATTTGTCGCTTCTTTACTCGC – 3'	1018	Ahmed, 2013
	SHV-R	5' – TTTATGGCGTTACCTTTGACC – 3'		
bla$_{CTX-M-1}$	CTX-M1F	5' – TGTTATTTCGTCTCTTTCAG – 3'	926	Wang, 2012
	CTX-M1R	5' –CATTCCCTTTCCGCTATTAC - 3'		
bla$_{CTX-M-2}$	CTX-M2F	5' - ACG CTA CCC CTG CTA TTT – 3'	986	Wang, 2012
	CTX-M2R	5' - GCT TTC CGC CTT CTG CTC – 3'		
bla$_{CTX-M-8}$	CTX-M8F	5'-CAGGAGTTTGAGATGATGAG -3'	910	Wang, 2012
	CTX-M8R	5'- GAGCGCTCCACATTTTTTAG-3'		
bla$_{CTX-M-9}$	CTX-M9F	5'-CGTATTGGGAGTTTGAGATG -3'	907	Wang, 2012
	CTX-M9R	5'-TTCAACAAAACCAGTTACAG -3'		
bla$_{CTX-M-25}$	CTX-M25F	5'- AGGATGATGAGAAAAAGCGT -3'	923	Wang, 2012
	CTX-M25R	5'- TACAAATAGTAAGTGGAGCG -3'		
bla$_{TEM}$	TEM-A2	5' – GTATCCGCTCATGAGACAAT – 3'	950	Dubois, 2005
	TEM-ext	5' –GTATATATGAGTAAACTTGGTCTG – 3'		
bla$_{GES}$	GES-F	5' – CTATTACTGGCAGGGATCG – 3'	594	Monteiro, 2012
	GES-R	5' – CCTCTCAATGGTGTGGGT – 3'		
bla$_{KPC}$	KPC-F	5' – TGTCACTGTATCGCCGTC – 3'	900	Doyle, 2012
	KPC-R	5' – CTCAGTGCTCTACAGAAAACC – 3'		
bla$_{IMP}$	IMP-F	5' – GAAGGCGTTTATGTTCATAC – 3'	587	Doyle, 2012
	IMP-R	5' – GTACGTTTCAAGAGTGATGC – 3'		
bla$_{VIM}$	VIM-F	5' - GTTTGGTCGCATATCGCAAC – 3'	389	Doyle, 2012
	VIM-R	5' – AATGCGCAGCACCAGGATAG – 3'		
bla$_{SPM}$	SPM-1F	5' – CTAAATCGAGAGCCCTGCTTG – 3'	850	Toleman, 2002
	SPM-1R	5' – CCTTTTCCGCGACCTTGATC – 3'		
bla$_{NDM}$	NDM-F	5'-GGTTTGGCGATCTGGTTTTC-3'	621	Poirel, 2011
	NDM-R	5'-CGGAATGGCTCATCACGATC-3'		

4.7. CARACTERIZAÇÃO CLONAL DOS ISOLADOS

A análise de tipificação dos isolados da ETE foi realizada através da técnica de ERIC-PCR. Os perfis gerados foram selecionados para avaliação por MLST.

4.7.1. *Enterobacterial Repetitive Intergenic Consensus* - ERIC-PCR

A técnica de ERIC-PCR foi utilizada no estudo para a análise do polimorfismo do DNA das amostras bacterianas com o objetivo de avaliar a similaridade genética entre os isolados. A extração de DNA genômico foi realizada utilizando o protocolo para Gram negative® Blood & Tissue (QiagenGmgH, Hilden, Alemanha) Dnaeasy kit de acordo com as instruções do fabricante. Para esta técnica foi utilizado o iniciador ERIC-2 (5'-AAGTAAGTGACTGGGGTGAGCG-3') com protocolo previamente descrito (VERSALOVIC; KOEUTH; LUPSKI, 1991). Os produtos da amplificação foram analisados por electroforese em gel de 1,5% de agarose em tampão TBE 0,5X por 1 hora, a 60 V e em seguida corados com brometo de etídio (3 mg / mL). O gel foi fotografado e analisado utilizando-se ImageQuant 300 imagens digitalizador (GE). Os Padrões de bandas foram analisados através do Programa BioNumerics versão 6.6 (Applied Maths, Kortrijk, Bélgica). O dendograma foi construído com a utilização do índice de Dice e o método "unweighted Pair Group Method with Arithmetic average" (UPGMA) (VAN BELKUM et al, 2007). Isolados bacterianos que apresentaram perfis de banda idênticos foram classificadas em um mesmo genótipo.

4.7.2. *Multilocus Sequence Typing* – MLST

A relação clonal dos isolados de *P. aeruginosa*, com um representante de cada perfil gerado pela ERIC-PCR, foi analisada pelo MLST. O protocolo utilizado para a determinação do perfil clonal por MLST encontra-se descrito no banco de

dados http://pubmlst.org/paeruginosa/. Os sete genes indicados pelo banco de dados são: *acsA*, *aroE*, *guaA*, *mutL*, *nuoD*, *ppsA* e *trpE* (Tabela 2).

Tabela 2. Funções e posições no genoma dos 7 genes utilizados no esquema de tipificação de *P. aeruginosa* por MLST

Local	Função do gene (cepa)	Posição no genoma de PAO1 (pares de bases)
acsA	Acetil coenzima A sintetase	969670
aroE	Chiquimato desidrogenase	26711
guaA	GMP sintase	4227237
mutL	DNA de proteínas de reparo “mismatch” (PA4946)	5551681
nuoD	NADH desidrogenase I cadeia C, D (PA2639)	2983963
ppsA	FosfoenolpiruvatoSintase (PA1770)	1914037
trpE	SintetaseAnthralite componente I (PA0609)	670980

Fonte: CURRAN e colaboradores, 2004.

4.7.2.1. *Amplificação e Sequenciamento dos Genes*

Sete pares de iniciadores utilizados para a amplificação e sequenciamento dos sete genes housekeeping (*acs*A, *aro*E, *gua*A, *mut*L, *nuo*D, *pps*A e *trp*E) foram descritos por L. Cacci (Manuscrito em preparação) utilizando o programa primer-blast (http: //www.ncbi. nlm.nih.gov/tools/primer-blast), com exceção da *acs*A-F (CURRAN et al, 2004).

Para amplificação, a mistura da reação foi preparada em um volume total de 25 µL contendo GoTaq® Colorless MasterMix (Promega Corporation, Madison, USA), 1 µM de cada iniciador e 1 µL da suspensão bacteriana. A amplificação de cada gene foi realizada em termociclador (Veriti 96 Well Thermal Cycler – Applied Biosystems) nas seguintes condições: ciclo inicial de 96ºC por 10 minutos, 35 ciclos de 96ºC por 1 minuto, anelamento na temperatura adequada a cada par de iniciadores por 30 segundos (Tabela 3), 72ºC por 1 minuto e um ciclo final de 72ºC por 7 minutos.

Os produtos da PCR foram purificados pelo Kit QIAquick PCR Purification (Qiagen®) e sequenciados em duplicata para cada iniciador, utilizando terminadores

dideoxi fluorescentes (BigDye; Applied Biosystems, Foster City, CA) em um sequenciador de DNA automatizado - Applied Biosystems ABI Prism 3730 (OTTO et al, 2008). O sequenciamento dos genes foi realizado na Plataforma de sequenciamento do PDTIS/FIOCRUZ.

Tabela 3. Temperatura de anelamento dos iniciadores utilizados no MLST

Gene alvo	Iniciadores	Tamanho (pb)	TA (ºC)
acsA	acsA-F acsA-R	520	53
aroE, amplificação (externo)	aroE-F aroE-R	867	55
aroE, sequenciamento (interno)	aroE-F aroE-R	676	50
guaA	guaA-F guaA-R	652	57
mutL	mutL-F mutL-R	530	53
nuoD	nuoD-F nuoD-R	600	53
ppsA	ppsA-F ppsA-R	578	50
trpE	trpE-F trpE-R	727	55

4.7.2.2. Análise Clonal

As sequências obtidas foram comparadas com as existentes no banco de dados de MLST para *P. aeruginosa* http://pubmlst.org/paeruginosa/ com a finalidade de determinar o perfil alélico e, subsequentemente, o tipo sequencial (ST) dos isolados e sua alocação nos complexos clonais existentes ou possível descrição de novos complexos. Para a análise filogenética os genes sequenciados foram concatenados e submetidos à análise filogenética usando o programa MEGA 5 (TAMURA et al, 2011).

5. RESULTADOS

5.1. PARÂMETROS FÍSICOS, QUÍMICOS E FÍSICO-QUÍMICOS DAS AMOSTRAS DOS PONTOS DE COLETA

O pH dos cinco pontos da primeira coleta foi mantido entre 6,0-6,8 enquanto que na segunda coleta ficou mantido entre 7,6-8,4 dentro da faixa de pH de crescimento de vários microrganismos, incluindo *Pseudomonas aeruginosa*. A turbidez mostrou concentrações elevadas no ponto 1 e ponto 2, nas duas coletas, principalmente devido à presença de sólidos em suspensão na água. Os níveis de oxigênio dissolvido foram maiores no ponto 2, onde ocorre a aeração e eleva os níveis de OD, o que é essencial para a biodegradação da matéria orgânica. A temperatura dos 5 pontos das duas coletas, manteve-se entre 28-30ºC, favorecendo a presença de formas mesofílicas. A concentração de cloro foi elevada no ponto 4, que é a etapa que o cloro líquido é adicionado ao tanque. Devido à presença de hipoclorito em alta concentração, a condutividade também foi alta nesse ponto, que é directamente proporcional à ionização de substâncias dissolvidas no líquido (Tabela 4).

Tabela 4. Parâmetros físicos, químicos e físico-químicos dos pontos de coleta da ETEH

Local (ponto)	Parâmetros abióticos													
	pH		Condutividade Sm/cm		Turbidez		DO mg/L		Temperatura ºC		Salinidade %		Cloro ppm	
	2008	2010	2008	2010	2008	2010	2008	2010	2008	2010	2008	2010	2008	2010
Gradeamento (1)	6.8	7.6	0.39	0.82	18	10	4.4	4.5	29	28	0.0	0.0	1	0.0
Aeração (2)	6.1	7.9	0.31	0.38	26	99	7.6	9.2	30	29	0.0	0.0	1	ND
Decantação (3)	6.1	8.2	0.29	0.38	14	6	1.5	9.3	30	29	0.0	0.0	1	ND
Cloração (4)	6.3	8.4	0.52	0.48	12	4	3.5	9.2	30	29	0.0	0.0	10	10.9
Efluente Tratado (5)	6.0	8.4	0.29	0.35	13	7	3.4	3.7	30	29	0.0	0.0	1	0.01

ND – Não Detectável. Os valores da escala foram estabelecidos de acordo com o manual dos equipamentos Horiba® e Extech®

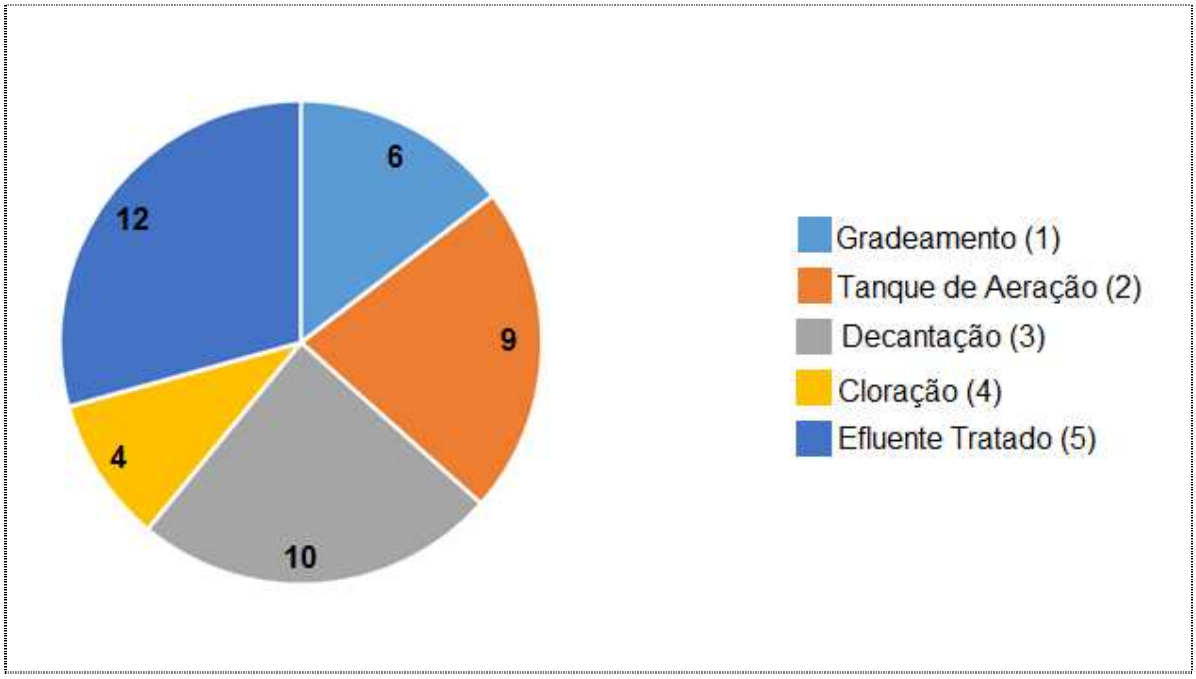
5.2. ISOLAMENTO E IDENTIFICAÇÃO DE *Pseudomonas aeruginosa*

5.2.1.Isolados da ETEH

Foram isoladas e identificadas, como *P. aeruginosa*, um total de 41 amostras dos 5 pontos da ETEH: Ponto 1 - gradeamento (n = 6); Ponto 2 - tanque de aeração (n = 9); Ponto 3 - tanque de decantação (n = 10); Ponto 4 - cloração (n = 4); Ponto 5 – efluente tratado (n = 12) (Gráfico 1).

Todos os isolados produziram colônias com pigmentos verdes e amareladas em agar cetrimide. Os isolados foram identificados bioquimicamente como *P. aeruginosa*. Os isolados bioquimicamente identificados como *P. aeruginosa*, bem como a cepa de referência de *P. aeruginosa* ATCC 29336, foram confirmados pela PCR de uma região espécifica do gene 16S rRNA para *P. aeruginosa* (956 pb). Os produtos da PCR da cepa de referência e de seis isolados foram sequenciados e apresentaram similaridade entre 97-99% com sequências do banco de dados Genbank.

Gráfico 1. Distribuição dos isolados de *P. aeruginosa* do ETEH pelos pontos de isolamento

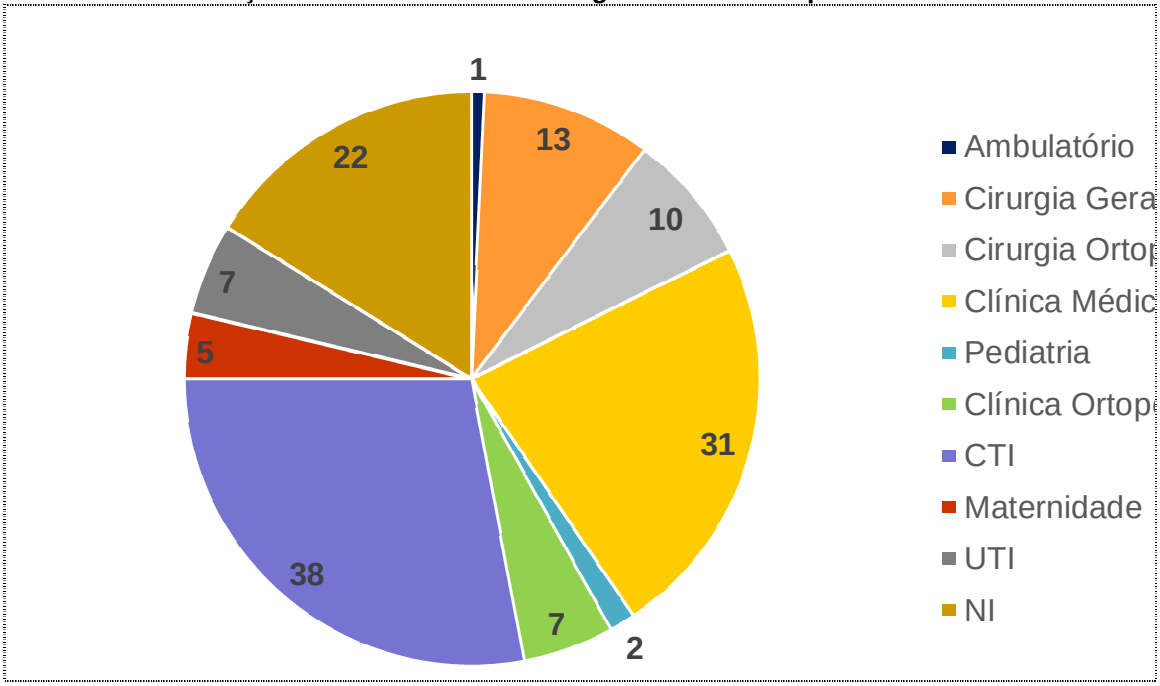


5.2.2.Isolados do HMLJ

Foram obtidos 136 isolados clínicos de *Pseudomonas aeruginosa* provenientes de infecções relacionadas à assistência à saúde (IRAS), distribuídos pelas áreas de isolamento do HMLJ: ambulatório (1), cirurgia geral (13), cirurgia ortopédica (10), clínica médica (31), pediatria (2), clínica ortopédica (7), CTI (37), maternidade (5), UTI (7), não informado-NI (23) (Gráfico 2).

Todos os isolados produziram colônias com pigmentos verdes e amareladas em ágar cetrimide. Os isolados foram identificados bioquimicamente como *P. aeruginosa*. Os isolados bioquimicamente identificados como *P. aeruginosa*, bem como a cepa de referência de *P. aeruginosa* ATCC 29336, foram confirmados por análise através da PCR e sequeciamento do gene 16S rRNA como descrito acima em 5.2.1.

Gráfico 2. Distribuição dos isolados de *P. aeruginosa* do HMLJ pela área de isolamento



CTI (Centro de Tratamento Intensivo); UTI (Unidade deTterapia Intensiva); NI (Não Informado)

5.3. SUSCEPTIBILIDADE ANTIMICROBIANA

5.3.1. Isolados da ETEH

Os 41 isolados foram testados frente a 17 antibióticos através do método de disco-difusão e apresentaram alta taxas de resistência aos vários antimicrobianos. Observou-se maior valor de resistência para fosfomicina (88%) seguido por ticarcillina/ácido clavulânico (71%), ceftriaxona (63%), aztreonam (59%), cefotaxima (54%), imipenema (39%), cefepima (24%), meropenema (22%), ceftazidima (20%), tobramicina (20%), polimixina B (20%), colistina (17%), gentamicina (15%), ciprofloxacina (12%), levofloxacina (12%), piperacillina/tazobactam (12%) e amicacina (2%). Níveis de resistência para fosfomicina, tobramicina, levofloxacina, ciprofloxacina e piperacillina/tazobactam foram menores nos 3 primeiros pontos da

estação de tratamento (gradeamento, tanque de aeração e tanque de decantação) com aumento significativo ($p<0.05$) nos pontos 4 e 5 (cloração e efluente tratado).

Dos 41 isolados, 14 (34%) apresentaram fenótipo de produtores de ESBL, 18 (44%) de KPC e 20 (49%) de MBL. Em 18 (44%) isolados foram detectados genes codificadores de ESBL e em 5 (12%) genes de carbapenemases. Trinta e quarto isolados (83%), distribuídos pelos pontos da ETEH, apresentaram perfil de multirresistência e foram classificados como MDR (82%) e XDR (18%) (Figura 2).

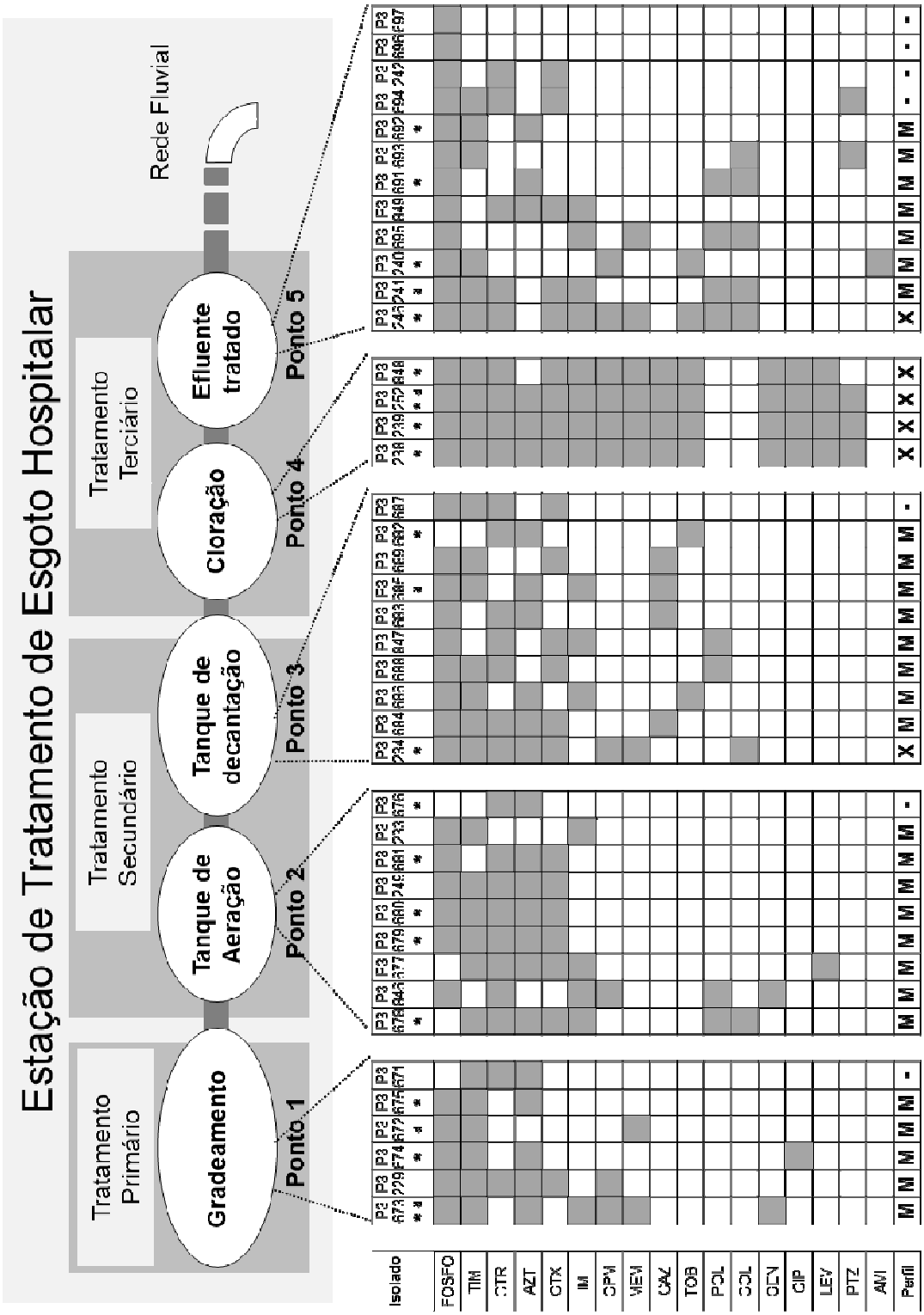


Figura 2.Susceptibilidade aos antibióticos dos isolados de *P. aeruginosa* dos 5 pontos daETEH
Quadrado preenchido–resistência aos antimicrobianos, quadrado vazio – susceptibilidade aos antimicrobianos, * - produtores de ESBL, ª – atividade carbapenemase, X - extensivamente droga resistente, M – multidroga resistência, — - não-MDR. FOSFO (fosfomicina), TIM (ticarcillina/ácido clavulânico), CTR (ceftriaxona), AZT (aztreonam), CTX (cefotaxima), IMI (imipenema), CPM (cefepima), MEM (meropenema), CAZ (ceftazidima), TOB (tobramicina), POL (polimixina), COL (colistina), GEN (gentamicina), CIP (ciprofloxacina), LEV (levofloxacina), PTZ (piperacillina/tazobactam), AMI (amicacina).

5.3.2. Isolados do HMLJ

Os 136 isolados foram testados frente a 17 antibióticos através do método de disco-difusão e apresentaram alta taxas de resistência aos vários antimicrobianos. Observou-se maior valor de resistência para fosfomicina (100%) seguido por cefotaxima (79%), ceftriaxona (76%), ciprofloxacina (50%), levofloxacina (49%), aztreonam (47%), ticarcillina/ácido clavulânico (46%), gentamicina (45%), tobramicina (41%), cefepima (37%), ceftazidima (35%), meropenema (33%), imipenema (30%), amicacina (27%), piperacillina/tazobactam (24%), colistina (8%) e polimixina B (7%) (Figura 3). Quando foi comparado o perfil de susceptibilidade dos isolados nos diferentes locais de isolamento do HMLJ, frente ao total de isolados, observamos uma diferença estatisticamente significativa ($p<0.05$) através do teste de Fisher entre os níveis de resistência para ticarcilina/ácido clavulânico (cirurgia geral), polimixina B e colistina (CTI), ciprofloxacina e levofloxacina (Maternidade) e meropenema e piperacilina/tazobactam (UTI) (Tabela 5).

Figura 3. Perfil de resistência aos antimicrobianos dos isolados de *P. aeruginosa* do HMLJ

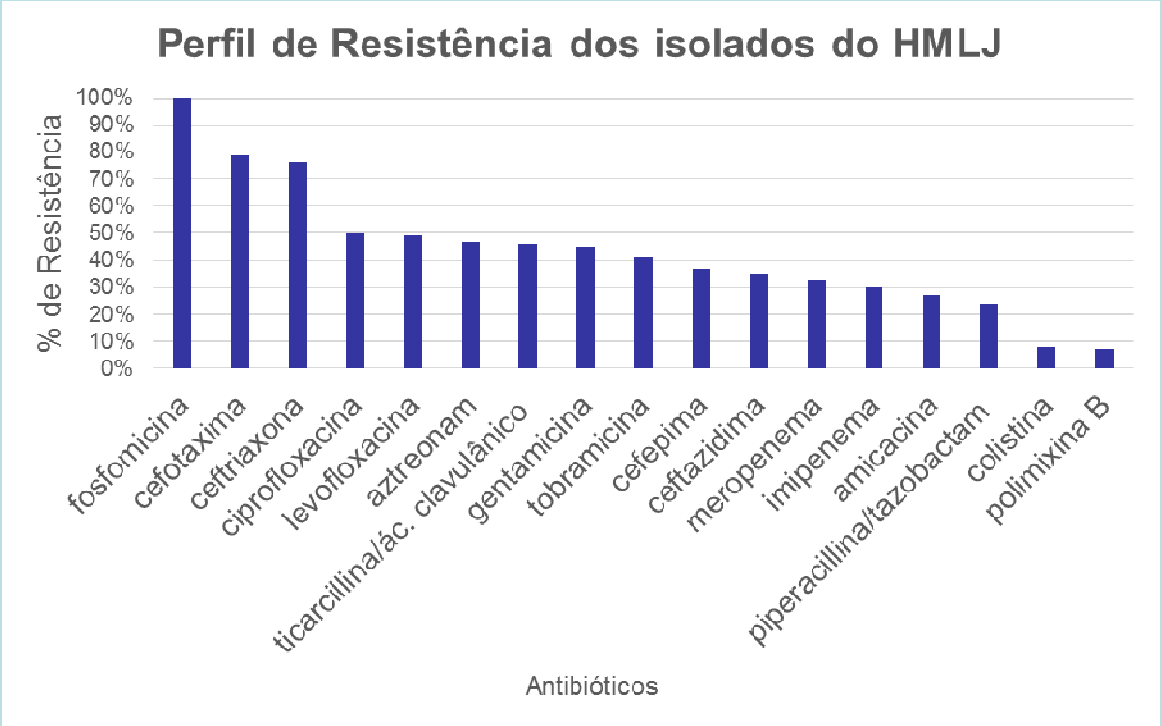


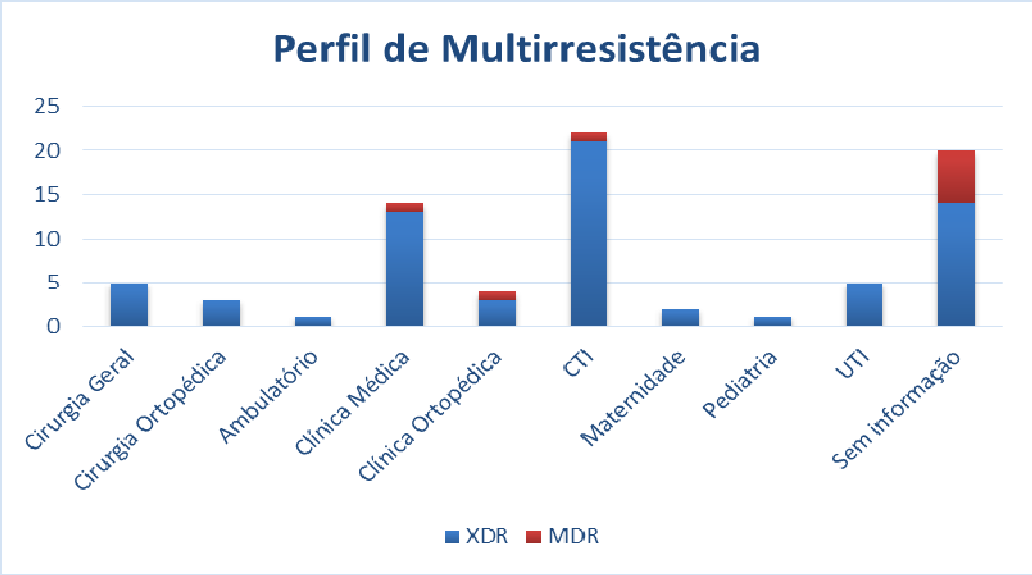
Tabela 5. Análise comparativa dos níveis de resistência entre os isolados de *P. aeruginosa* de cada local de isolamento e o total de isolados do HMLJ

Antibiótico	Nível de significância de p<0.05 [a]							
	Cirurgia		Clínica médica	CTI	Maternidade	Ortopedia	Pediatria	UTI
	Geral	Ortopédica						
FOSFO	ns	ns	ns	ns	ns	Ns	ns	ns
TIM	**0,0316***	0,3168	0,2729	0,9674	0,7805	0,8576	0,9175	0,1946
CTR	0,9706	0,2434	0,9102	0,6599	0,3978	0,1452	0,4337	0,5713
AZT	0,0967	0,2961	0,2423	0,6430	0,5692	0,8280	0,9340	0,2082
CTX	0,8327	0,1521	0,8776	0,4016	0,2570	0,1806	0,4723	0,6858
IMI	0,2617	0,1743	0,6319	0,7891	0,6383	0,9293	0,3543	0,9293
COM	0,6674	0,2852	0,8936	0,4719	0,8829	0,6603	0,2829	0,0660
MEM	0,1894	0,1292	0,5513	0,6986	0,7474	0,8040	0,3217	**0,0380***
CAZ	0,1463	0,7346	0,9840	0,5565	0,8289	0,2533	0,6661	0,0535
TOB	0,4647	0,4869	0,3593	0,0908	0,0645	0,9298	0,2390	0,1145
POL	0,9643	0,3742	0,1194	**0,0361***	0,5293	0,4569	0,6904	0,4569
COL	0,9600	0,3496	0,1013	**0,0195***	0,5077	0,4335	0,6750	0,4335
GEN	0,3279	0,3609	0,7679	0,1985	0,8302	0,9175	0,2048	0,1689
CIP	0,7910	0,2219	0,4175	0,1889	**0,0279***	0,7124	0,1602	0,2687
LEV	0,4564	0,2392	0,4610	0,1638	**0,0302***	0,7408	0,1664	0,2527
PTZ	0,1889	0,7988	0,4300	0,2702	0,2173	0,1452	0,4337	**0,0047***
AMI	0,7482	0,6191	0,2683	0,2085	0,1744	0,9369	0,4737	0,9369

FOSFO (fosfomicina), TIM (ticarcillina/ácido clavulânico), CTR (ceftriaxona), AZT (aztreonam), CTX (cefotaxima), IMI (imipenema), CPM (cefepima), MEM (meropenema), CAZ (ceftazidima), TOB (tobramicina), POL (polimixina), COL (colistina), GEN (gentamicina), CIP (ciprofloxacina), LEV (levofloxacina), PTZ (piperacilina/tazobactam), AMI (amicacina). [a] p<0.05 para a análise comparativa da resistência através do teste de Fisher entre os isolados de cada local de isolamento com total de isolados do HMLJ; ns (não foi possível calcular) * (aumento significativo de resistência entre os pontos comparados).

Dos 136 isolados, 59 (43%) apresentaram fenótipo ESBL, 53 (39%) fenótipo KPC e 64 (47%) fenótipo MBL. Setenta e seis isolados (56%) produziram ESBL e 7 (5%) apresentaram atividade carbapenemase. Setenta e sete isolados (57%), de diferentes unidades do HMLJ, apresentaram perfil de multirresistência e foram classificados como MDR (12%) e XDR (88%) (Figura 4).

Figura 4. *P. aeruginosa* multirresistentes aos antibióticos isoladas do HMLJ distribuídas pelas unidades do hospital

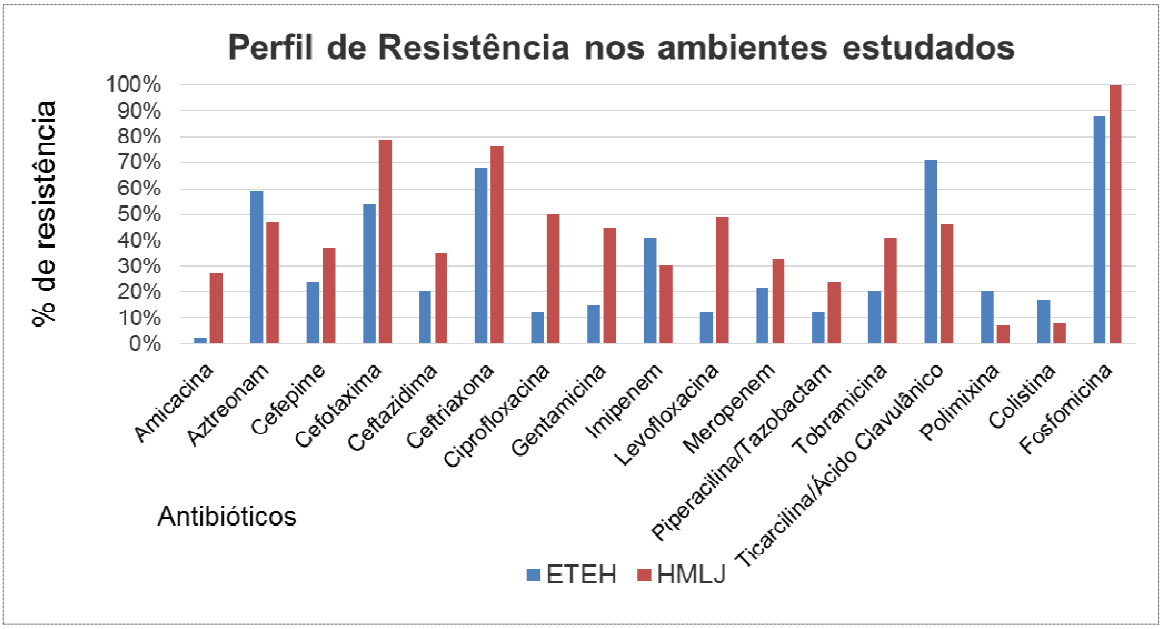


MDR (multidroga resistente); XDR (extensivamente droga resistente)

5.3.3. Análise comparativa – isolados da ETEH e HMLJ

Pela análise da susceptibilidade entre todos os isolados de *P. aeruginosa* (clínicos e do efluente), foi verificada maior resistência à fosfomicina (97%) seguido por ceftriaxona (75%) e cefotaxima (73%) (Figura 5).

Figura 5. Perfil de resistência aos antimicrobianos de *P. aeruginosa* nos ambientes estudados - ETEH e HMLJ



Foi observada uma diferença estatisticamente significativa ($p<0.05$) entre os níveis de resistência para ticarcillina/ácido clavulânico, cefotaxima, tobramicina, polimixina, gentamicina, ciprofloxacina, levofloxacina e amicacina dos isolados clínicos e do efluente hospitalar (Tabela 6).

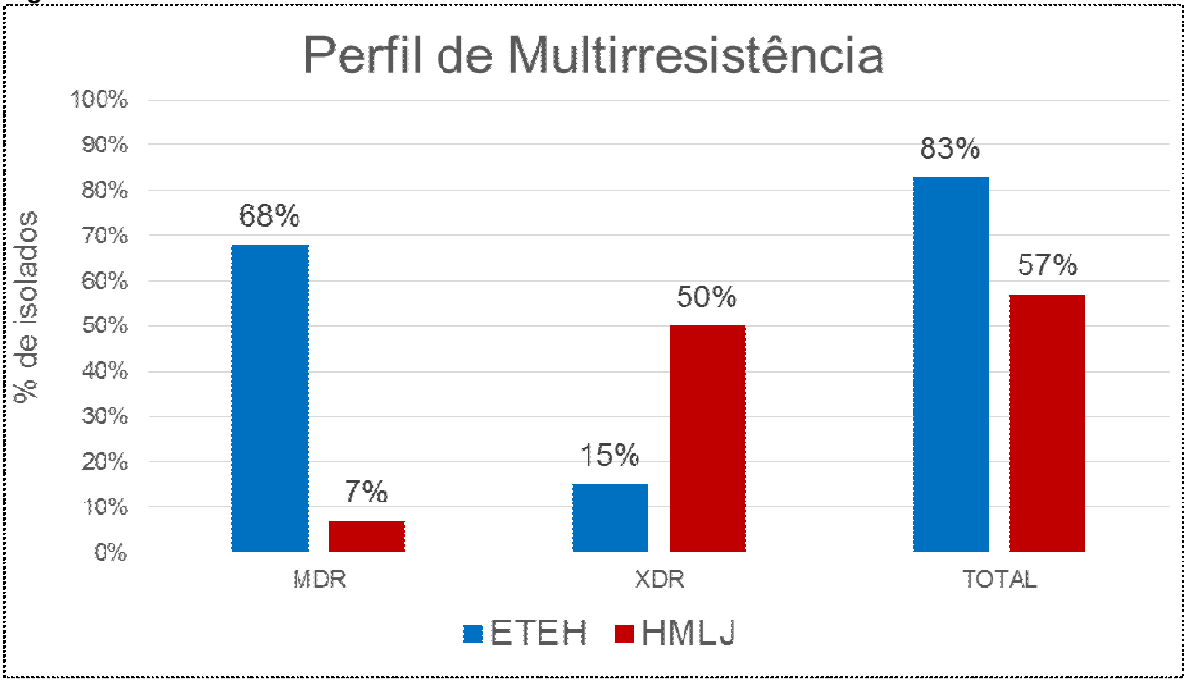
Tabela 6. Prevalência da Resistência antimicrobiana nos isolados de *P. aeruginosa* da ETEH e HMLJ

ATB	Número e (%) de isolados resistentes			p<0.05 [a]
	ETEH (n=*41*)	HMLJ (n=*136*)	TOTAL (N=*177*)	
FOSFO	36 (88)	136 (100)	172 (97)	Ns
TIM	29 (71)	63 (46)	92 (52)	0,006105*
CTR	28 (68)	104 (76)	132 (75)	0,29182
AZT	24 (59)	64 (47)	88 (50)	0,197588
CTX	22 (54)	108 (79)	130 (73)	0,001064*
IMI	17 (41)	41 (30)	58 (33)	0,175987
COM	10 (24)	50 (37)	60 (34)	0,142307
MEM	9 (22)	45 (33)	54 (31)	0,174595
CAZ	8 (20)	48 (35)	56 (32)	0,056822
TOB	8 (20)	56 (41)	64 (36)	0,011379*
POL	8 (20)	10 (7)	18 (10)	0,023947*
COL	7 (17)	11 (8)	18 (10)	0,095215
GEN	6 (15)	61 (45)	67 (38)	0,000471*
CIP	5 (12)	68 (50)	73 (41)	1,63E-05*
LEV	5 (12)	67 (49)	72 (41)	2,28E-05*
PTZ	5 (12)	32 (24)	37 (21)	0,117699
AMI	1 (2)	37 (27)	38 (21)	0,000711*

ATB (antimicrobianos): FOSFO (fosfomicina), TIM (ticarcillina/ácido clavulânico), CTR (ceftriaxona), AZT (aztreonam), CTX (cefotaxima), IMI (imipenema), CPM (cefepima), MEM (meropenema), CAZ (ceftazidima), TOB (tobramicina), POL (polimixina), COL (colistina), GEN (gentamicina), CIP (ciprofloxacina), LEV (levofloxacina), PTZ (piperacillina/tazobactam), AMI (amicacina).
[a] p<0.05 para a análise comparativa da resistência através do teste de Fisher entre o total de isolados da ETEH e HMLJ; ns (não foi possível calcular) * (aumento significativo de resistência entre os pontos comparados)

Os isolados de *P. aeruginosa* multirresistentes foram mais frequentes na ETEH (83%) do que no HMLJ (57%). Em relação ao perfil de multidroga resistência (MDR), os isolados da ETEH tiveram um aumento significativo (p<0.05) em relação aos isolados do HMLJ (1,6921E-17). No entanto, em relação ao perfil de extensivamente droga resistente (XDR), foi observado um aumento significativo entre os isolados do HMLJ (p<0.05) em relação aos isolados da ETEH (5,7129E-05) (Figura 6).

Figura 6. Perfil de Multirresistência dos isolados da ETEH e do HMLJ



MDR (multidroga resistente); XDR (extensivamente droga resistente)

5.4. DETECÇÃO DE GENES QUE CODIFICAM ESBL, KPC E MBL

5.4.1. Isolados da ETEH

Dos 41 isolados da ETEH, 14 (34%) demonstraram fenótipo de produtores de ESBL, 13 apresentaram o gene *bla*$_{TEM}$ (45%), sete *bla*$_{SHV}$ (24%) e um *bla*$_{CTX-M-1}$ (4%). Dezoito (44%) isolados demonstraram fenótipo de produtores de KPC, 14 apresentaram o gene *bla*$_{KPC}$ (37%). Dos 20 (49%) isolados que demonstraram fenótipo para MBL, 14 (41%) apresentaram gene *bla*$_{VIM}$ e 6 o gene *bla*$_{SPM}$ (18%) (Tabela 7).

Tabela 7. Genes de beta-lactamases detectados nos isolados de *P. aeruginosa* da ETEH

Isolados	Perfil Fenotípico			Genes de beta-lactamases	Ponto de Isolamento
	ESBL	KPC	MBL		
P3229	+	-	-	*bla*$_{SHV}$, *bla*$_{KPC}$	1
P3233	-	+	+	-	2
P3234	+	+	-	*bla*$_{TEM}$	3
P3238	+	+	+	*bla*$_{TEM}$, *bla*$_{VIM}$, *bla*$_{SPM}$	4
P3239	+	+	+	*bla*$_{TEM}$, *bla*$_{VIM}$	4
P3240	+	-	-	*bla*$_{TEM}$, *bla*$_{KPC}$, *bla*$_{VIM}$	5
P3241	-	+	+	-	5
P3242	-	-	-	-	5
P3246	+	+	+	*bla*$_{TEM}$	5
P3249	-	-	-	*bla*$_{SHV}$, *bla*$_{VIM}$	2
P3252	+	+	+	*bla*$_{SHV}$, *bla*$_{KPC,}$ *bla*$_{VIM}$	4
P3671	-	-	-	-	1
P3672	-	+	+	*bla*$_{SPM}$	1
P3673	+	+	+	*bla*$_{SPM}$	1
P3674	-	-	-	*bla*$_{SHV}$, *bla*$_{SPM}$	1
P3675	-	-	-	*bla*$_{VIM}$	1
P3676	-	-	-	*bla*$_{TEM}$	2
P3677	-	+	+	*bla*$_{KPC}$, *bla*$_{VIM}$	2
P3678	-	+	+	*bla*$_{CTX-M-1}$, *bla*$_{SHV}$, *bla*$_{KPC}$	2
P3679	-	-	-	*bla*$_{KPC}$	2
P3680	-	-	-	*bla*$_{KPC}$	2
P3681	-	-	-	*bla*$_{KPC}$	2
P3682	-	-	-	*bla*$_{KPC}$	3
P3683	+	-	+	-	3
P3684	+	-	+	-	3
P3685	+	+	+	-	3
P3686	-	+	+	*bla*$_{TEM}$	3
P3687	-	-	-	*bla*$_{KPC}$	3
P3688	-	-	-	*bla*$_{KPC}$	3
P3689	+	-	+	*bla*$_{TEM}$, *bla*$_{KPC}$	3
P3691	-	-	-	*bla*$_{TEM}$	5
P3692	-	-	-	*bla*$_{TEM}$, *bla*$_{VIM}$	5
P3693	-	-	-	*bla*$_{VIM}$	5
P3694	-	-	-	*bla*$_{VIM}$, *bla*$_{SPM}$	5
P3695	-	+	+	*bla*$_{VIM}$, *bla*$_{SPM}$	5
P3696	-	-	-	-	5
P3697	-	-	-	-	5
P3846	+	+	+	*bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{KPC,}$ *bla*$_{VIM}$	2
P3847	-	+	+	*bla*$_{KPC,}$ *bla*$_{VIM}$	3
P3848	+	+	+	*bla*$_{TEM}$, *bla*$_{VIM}$	4
P3849	-	+	+	*bla*$_{TEM}$, , *bla*$_{SHV}$	5

Pontos de isolamento: (1) Gradeamento/ afluente; (2) Tanque de Aeração; (3) Tanque de Decantação; (4) Cloração; (5) Efluente Tratado.

5.4.2. Isolados do HMLJ

Dos 136 isolados do HMLJ, 59 (43%) demonstraram fenótipo de produtores de ESBL, 16 apresentaram gene *bla*$_{TEM}$ (22%), 10 o gene *bla*$_{CTX-M-2}$ (14%), e um pelo gene *bla*$_{SHV}$ (1,4%). Cinquenta e três (39%) dos isolados demonstraram fenótipo de produtores de KPC, 18 apresentaram o gene *bla*$_{KPC}$ (20%). Dos 64 (47%) isolados que apresentaram fenótipo de MBL, em 7 (9%) foram detectados o gene *bla*$_{VIM}$ e em 3, o gene *bla*$_{SPM}$ (4%).

5.4.3. Análise comparativa – isolados da ETEH e HMLJ

Comparando o percentual de detecção dos genes que codificam beta-lactamases, nos dois ambientes estudados, foi verificado que nos isolados da ETEH o percentual foi maior para todos os genes em relação aos isolados do HMLJ, com exceção do gene *bla*$_{CTX-M-1}$ que foi detectado apenas na ETEH e do gene *bla*$_{CTX-M-2}$ encontrado nos isolados clínicos (Tabela 8). Os genes *bla*$_{PER}$, *bla*$_{VEB}$, *bla*$_{CTX-M-8}$, *bla*$_{CTX-M-9}$, *bla*$_{CTX-M-25}$, *bla*$_{GES}$, *bla*$_{IMP}$, e *bla*$_{NDM}$ não foram encontrados em qualquer dos isolados de *P. aeruginosa*.

Tabela 8. Distribuição dos genes de resistência aos antibióticos nos isolados da ETEH e HMLJ

Local de Isolamento	**Perfil Fenotípico (n/%)**			**Genes de beta-lactamases (n/%)**						
	ESBL	**KPC**	**MBL**	*bla* CTX-M1	*bla* CTX-M2	*bla* TEM	*bla* SHV	*bla* KPC	*bla* VIM	*bla* SPM
ETEH (n=41)	14/34	18/44	20/49	1/4	0/0	13/45	7/24	14/37	14/41	6/18
HMLJ (n=136)	59/43	53/39	64/47	0/0	10/14	16/22	1/1	18/20	7/9	3/4

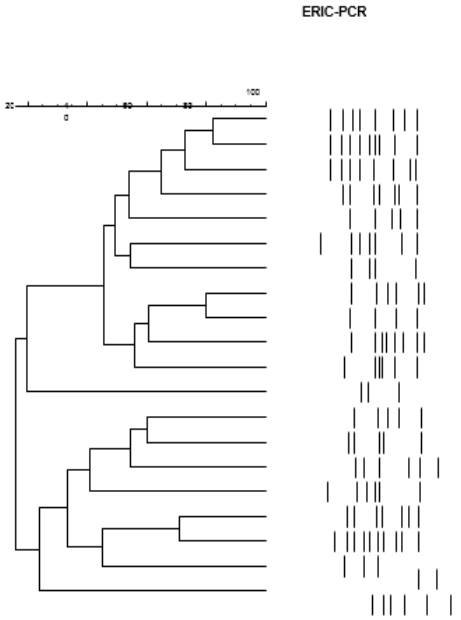
5.5. CARACTERIZAÇÃO CLONAL DOS ISOLADOS DE *P. aeruginosa*

5.5.1. ERIC-PCR

5.5.1.1. Isolados da ETEH

A variabilidade genética dos 177 isolados de *Pseudomonas aeruginosa* foi analisada através da técnica de ERIC-PCR que mostrou alta diversidade genética (<80% de similaridade). Entre as 41 cepas isoladas de diferentes pontos da ETEH, a ERIC-PCR agrupou 20 perfis distintos. Os padrões de bandas foram altamente reprodutíveis em análise visual e automatizado (Figura 7).

Figura 7. Perfil de bandas representativo dos 20 perfis obtidos para os isolados de *P. aeruginosa* da ETEH por ERIC2-PCR



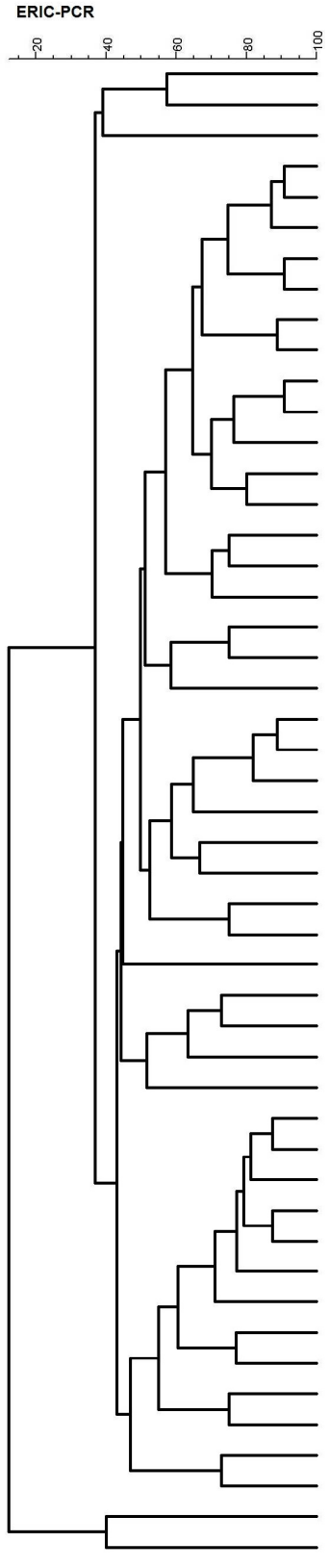
Perfis	nº de isolados	Data	Perfil de Resistência	Ponto
7	2	2010	MDR (2)	1(2)
8	1	2010	MDR (1)	1
5	1	2008	XDR (1)	5
2	2	2008	MDR (2)	2
6	4	2010	MDR (2)	1, 2(3)
1	4	2008	MDR (2), XDR (1)	1, 3, 5(2)
3	3	2008	XDR (3)	4(3)
13	7	2010	MDR (7)	3(7)
9	1	2010	MDR (1)	1
11	1	2010	MDR	2
12	1	2010	MDR	2
10	1	2010	MDR	2
18	1	2010	-----	5
19	1	2008	MDR	2
17	4	2010	MDR (2)	5(4)
20	3	2008	MDR (2), XDR (1)	3(1), 4(1), 5(1)
14	1	2010	------	3
15	1	2010	MDR	5
16	1	2010	MDR	5
4	1	2008	MDR (1)	5

Dendrograma obtido com base no agrupamento UPGMA e análise de similaridade com cálculo do coeficiente de Dice. Análise realizada utilizando-se o programa BioNumerics 6.6 (Applied Maths, Sint-Martens-Latem, Belgium).

5.5.1.2. Isolados do HMLJ

Entre os 136 isolados clínicos de *Pseudomonas aeruginosa*, distribuídos pelas áreas de isolamento do HMLJ, a ERIC-PCR agrupou 49 perfis distintos. Os padrões de bandas foram altamente reprodutíveis em análise visual e automatizado (Figura 8).

Figura 8. Perfil de bandas representativo dos 49 perfis obtidos para os isolados de *P. aeruginosa* do HMLJ por ERIC2-PCR



Perfil	nº de isolados	Perfil Resistência
47	6	XDR(6)
10	1	XDR(1)
2	1	-
19	15	MDR(1),XDR(3)
23	3	-
18	4	MDR(1),XDR(3)
17	8	XDR(2)
20	3	-
22	2	XDR(1)
21	2	XDR(2)
16	2	XDR(1)
15	4	XDR(2)
27	1	XDR(1)
13	5	XDR(4)
14	1	-
26	1	-
25	2	XDR(2)
36	1	-
42	2	XDR(1)
41	4	XDR(3)
28	5	XDR(4)
29	1	MDR(1)
31	13	MDR(1),XDR(7)
30	1	MDR(1)
33	3	MDR(1),XDR(2)
38	1	-
37	2	XDR(2)
39	1	XDR(1)
40	1	-
44	3	XDR(1)
34	1	MDR(1)
35	1	-
32	1	XDR(1)
43	1	-
7	1	XDR(1)
4	1	XDR(1)
6	2	XDR(2)
8	5	XDR(2)
9	1	-
3	8	MDR(1),XDR(4)
5	2	XDR(1)
11	1	XDR(1)
12	1	XDR(1)
24	5	MDR(1),XDR(3)
1	2	-
46	1	XDR(1)
45	2	-
48	1	XDR(1)
49	1	XDR(1)

Dendrograma obtido com base no agrupamento UPGMA e análise de similaridade com cálculo do coeficiente de Dice. Análise realizada utilizando-se o programa BioNumerics 6.6 (Applied Maths, Sint-Martens-Latem, Belgium).

5.5.2. MLST

5.2.2.1. Isolados da ETEH

Análise por MLST dos 20 perfis distintos definidos através da ERIC-PCR da ETEH, revelou um total de 15 ST diferentes, com cinco previamente descritos (ST238, ST244, ST381, ST595 e ST1621) e dez ST novos, que foram depositados na base de dados do MLST para *P. aeruginosa* (ST1853, ST1854, ST1855, ST1856, ST1857, ST1858, ST1859, ST1860, ST1861, ST1862). O poder discriminatorio da ERIC-PCR foi maior do que o MLST, onde cepas pertencentes ao mesmo ST apresentaram diferentes perfis de ERIC (Tabela 9).

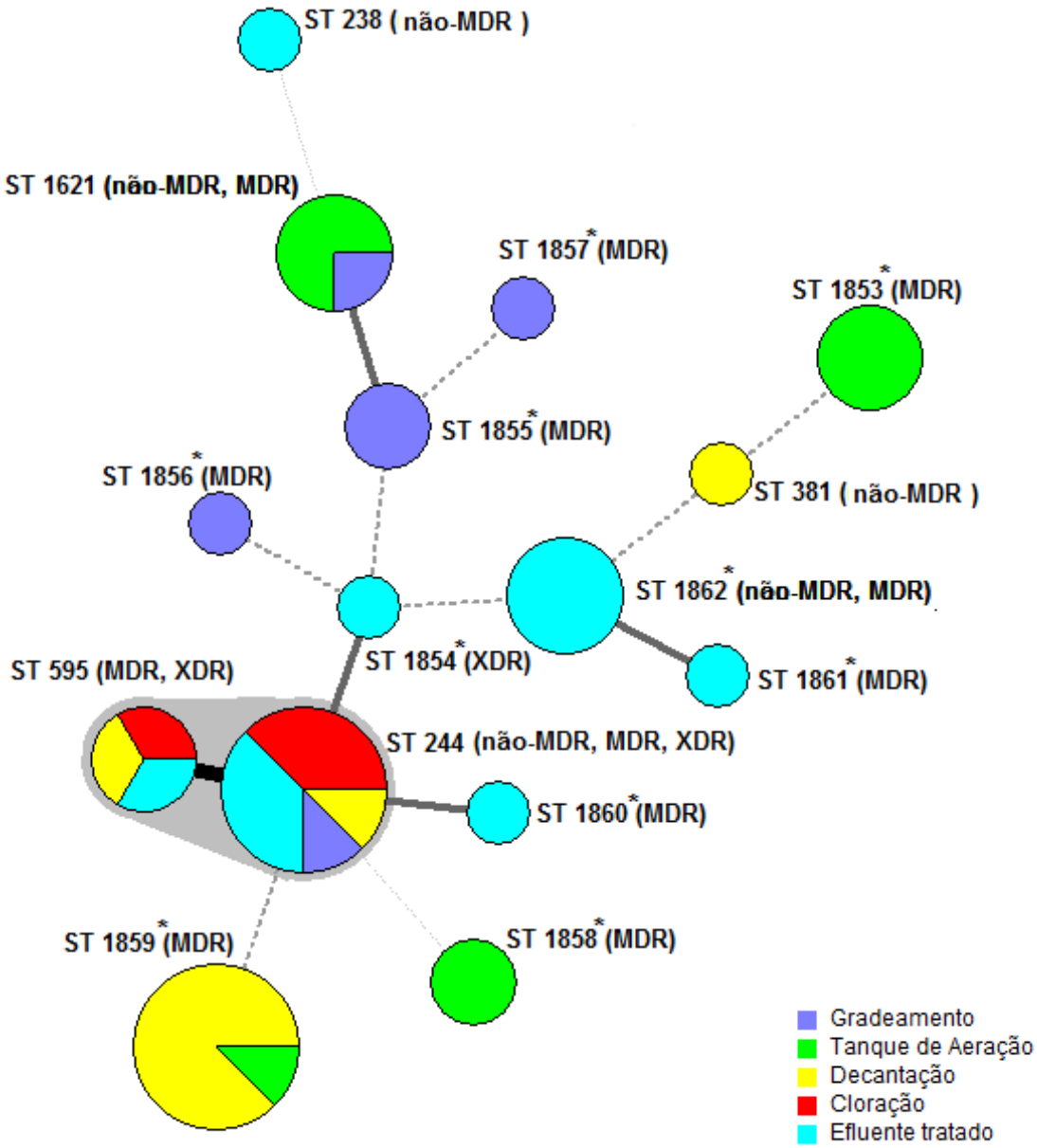
Tabela 9. Perfis alélicos por MLST dos 20 perfis distintos da ERIC-PCR para os isolados de *P. aeruginosa* da ETEH

Perfil ERIC	Nº de isolados	Ponto Coleta	ST	Perfil alélico						
				*acs*A	*aro*E	*gua*A	*mut*L	*nuo*D	*pps*A	*trp*E
1	4	1, 3, 5	244	17	5	12	3	14	4	7
2	2	2	1853	16	22	11	5	4	4	10
3	3	4	244	17	5	12	3	14	4	7
4	1	5	244	17	5	12	3	14	4	7
5	1	5	1854	17	5	44	110	14	81	7
6	4	1, 2	1621	5	54	99	48	1	6	3
7	2	1	1855	5	54	44	48	14	81	3
8	1	1	1856	30	2	44	110	1	97	7
9	1	1	1857	28	54	44	110	4	79	3
10	1	2	1858	17	2	11	3	81	38	3
11	1	2	1859	17	5	36	7	27	4	5
12	1	2	1858	17	2	11	3	81	38	3
13	7	3	1859	17	5	36	7	27	4	5
14	1	3	381	11	20	1	65	4	4	10
15	1	5	1860	17	5	44	3	14	77	5
16	1	5	1861	17	20	49	110	11	79	10
17	4	5	1862	17	20	12	110	4	81	10
18	1	5	238	5	1	59	6	1	33	42
19	1	2	1853	16	22	11	5	4	4	10
20	3	3, 4, 5	595	17	5	12	5	14	4	7

Os dados gerados a partir da árvore de *Minimum Spanning Tree* (MST), dos isolados da ETEH, mostraram que os organismos não-MDR pertencem aos ST238 e ST381, com a exceção dos ST244, ST1621 e ST1862 que agruparam outros isolados multirresistentes. Os ST244 e ST1859 (descrito neste estudo) foram representados por um maior número de isolados (n = 8), cada um. O ST244 esteve presente em 4 dos 5 pontos da ETEH, com maior frequência nas etapas de cloração e efluente tratado, o ST595 em 3 pontos, com isolados igualmente distribuidos nas etapas de decantação, cloração e efluente tratado e o ST1859 em dois pontos com maior frequência no ponto de decantação.

Entre os novos ST, nove foram associados a pelo menos uma cepa MDR, e um isolado pertencente ao ST1854 apresentou perfil XDR. Os demais isolados XDR foram detectados nos ST244 e ST595. Os ST595 e ST244 por compartilharem cinco alelos idênticos foram agrupados em um mesmo Complexo Clonal (CC) (Figura 9).

Figura 9. *Minimum Spanning Tree* (MST) dos ST do MLST de *Pseudomonas aeruginosa* isolados da ETEH



A árvore foi baseada na análise do perfil alélico dos genes *acs*A, *aro*E, *gua*A, *mut*L, *nuo*D, *pps*A and *trp*E. Isolados não Multidroga Resistente (não-MDR), Multidroga Resistente (MDR) e Extensivamente Droga Resistente (XDR) foram indicados ao lado de cada ST. Círculos indicam o ST específico e o número de isolados de acordo com o tamanho do círculo. As cores dos círculos indicam o ponto de coleta na ETEH. Novos ST estão marcados com (*). Linhas que ligam os ST, indicam que eles diferem em um alelo (linha grossa sólida), ou 3 a 5 locus (linhas finas e pontilhadas). O halo que circula o ST-244 e ST-595 indica a formação de um complexo clonal (CC244). A relação entre os ST foi determinada pela análise com BioNumerics (BioNumerics 6.6) onde o CC foi formado com ST com um ou dois loci variáveis.

5.2.2.2. Isolados do HMLJ

Análise por MLST dos 49 perfis distintos do HMLJ, definidos pela ERIC-PCR revelou um total de 36 ST diferentes, nove previamente descritos (ST111, ST244, ST253, ST261, ST274, ST304, ST309, ST1247 e ST1284) e 27 novos, que foram depositados na base de dados do MLST para *P. aeruginosa* (ST1932, ST1933, ST1934, ST1935, ST1936, ST1937, ST1938, ST1939, ST1940, ST1941, ST1942, ST1944, ST1945, ST1946, ST1947, ST1948, ST1949, ST1951, ST1952, ST1953, ST1954, ST1955, ST1957, ST1958, ST1959, ST1961 e ST1962) (Tabela 10).

Tabela 10. Perfis alélicos por MLST dos 49 perfis distintos da ERIC-PCR para os isolados de *P. aeruginosa* do HMLJ

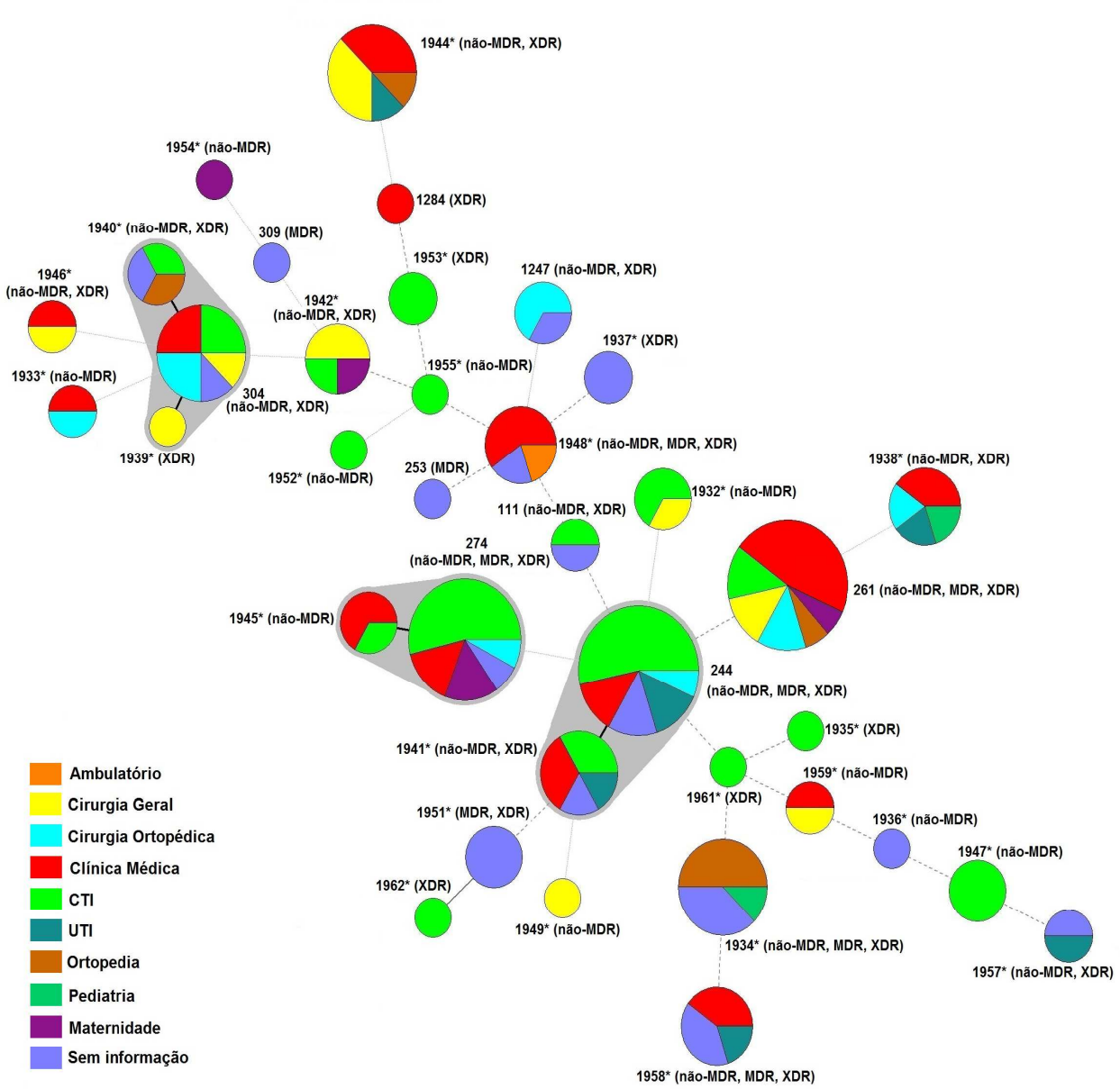
Perfil ERIC	Nº de isolados	Ponto Coleta	ST	Perfil alélico						
				***acs*A**	***aro*E**	***gua*A**	***mut*L**	***nuo*D**	***pps*A**	***trp*E**
1	2	1	1932	45	5	15	35	17	8	177
2	1	2	1933	142	12	65	6	1	16	125
3	8	3,4,10	1934	15	5	36	140	27	4	2
4	1	1	1935	15	21	36	31	4	15	8
5	1	10	1936	99	19	19	15	4	4	3
6	2	10	1937	99	162	7	7	1	3	3
7	1	1	304	82	11	3	13	1	2	4
8	5	2,4,5,6	1938	146	122	1	18	3	4	3
9	1	5	1933	142	12	65	6	1	16	125
10	1	7	1939	82	11	3	13	1	2	163
11	1	1	1940	82	93	3	13	1	2	4
12	1	10	1941	17	35	12	3	14	4	7
13	5	1,2,5,7	304	82	11	3	13	1	2	4
14	1	7	1932	45	5	15	35	17	8	177
15	4	1,7,8	1942	82	16	5	21	1	19	15
16	2	1,10	111	17	5	5	4	4	4	3
17	8	3,5,6,7	1944	9	8	5	73	95	20	9
18	4	5,6,10	1958	39	35	9	140	27	5	2
19	15	1,2,3,5,7,8	261	105	5	30	3	3	4	14
20	3	1,5	1945	23	35	11	7	1	12	7
21	2	5,10	304	82	11	3	13	1	2	4
22	2	5,7	1946	71	30	64	26	48	59	143
23	3	1	1947	99	6	19	11	4	15	9
24	5	5,9,10	1948	99	5	5	22	1	6	3
25	2	1,2	244	17	5	12	3	14	4	7
26	1	7	1949	70	35	12	119	3	1	18
27	1	3	1940	82	93	3	13	1	2	4
28	5	1,5,6	1941	17	35	12	3	14	4	7
29	1	10	253	4	4	16	12	1	6	3
30	1	10	244	17	5	12	3	14	4	7
31	13	1,2,5,8,10	274	23	5	11	7	1	12	7
32	1	5	244	17	5	12	3	14	4	7
33	3	10	1951	88	35	28	3	4	13	7
34	1	10	309	13	8	9	3	1	17	15
35	1	1	1952	33	4	57	62	1	1	26
36	1	10	1940	82	93	3	13	1	2	4
37	2	1	1953	12	93	5	21	5	6	118
38	1	8	1954	81	4	13	3	93	17	13
39	1	5	1284	32	8	5	3	5	6	26
40	1	1	1955	2	4	5	21	1	6	11
41	4	1	244	17	5	12	3	14	4	7
42	2	6,10	1957	99	57	7	21	4	15	1
43	1	5	1958	39	35	9	140	27	5	2
44	3	2,10	1247	15	5	77	72	3	6	68
45	2	5,7	1959	99	4	11	31	4	4	113
46	1	10	244	17	5	12	3	14	4	7
47	6	1,5,6	244	17	5	12	3	14	4	7
48	1	1	1961	15	5	29	31	4	4	7
49	1	1	1962	88	27	3	31	4	13	7

Ponto de isolamento:1-CTI; 2- Cirurgia ortopédica; 3- ortopedia; 4-pediatria;5-clínica médica; 6- UTI; 7-cirurgia geral; 8-maternidade; 9-ambulatório; 10-sem informação.

Os dados gerados a partir da árvore de MST, dos isolados do HMLJ, demonstraram que as cepas não-MDR pertencentes aos ST (1932, 1933, 1936, 1945, 1947 1949, 1952, 1954, 1955 e 1959) estiveram presentes em 5 dos 10 locais de isolamento das linhagens do HMLJ. Os ST 244 e 261 foram representados por um maior número de isolados (n = 15) para cada. O ST261 foi detectado em 7 das 10 unidades avaliadas, com maior incidência na clínica médica. O ST 244 esteve presente em 5 unidades, com maior incidência no CTI.

Entre os novos ST, 4 foram associados a pelo menos um isolado MDR, e 17 foram associados a pelo menos um isolado XDR. Os ST que compartilharam pelo menos cinco alelos idênticos foram agrupados no mesmo Complexo Clonal (CC): CC304 (ST304, ST1939 e ST1940), CC274 (ST274 e ST1945) e CC244 (ST244 e ST1941) (Figura 10).

Figura 10. *Minimum spanning tree* (MST) dos ST do MLST de *Pseudomonas aeruginosa* isolados do HMLJ



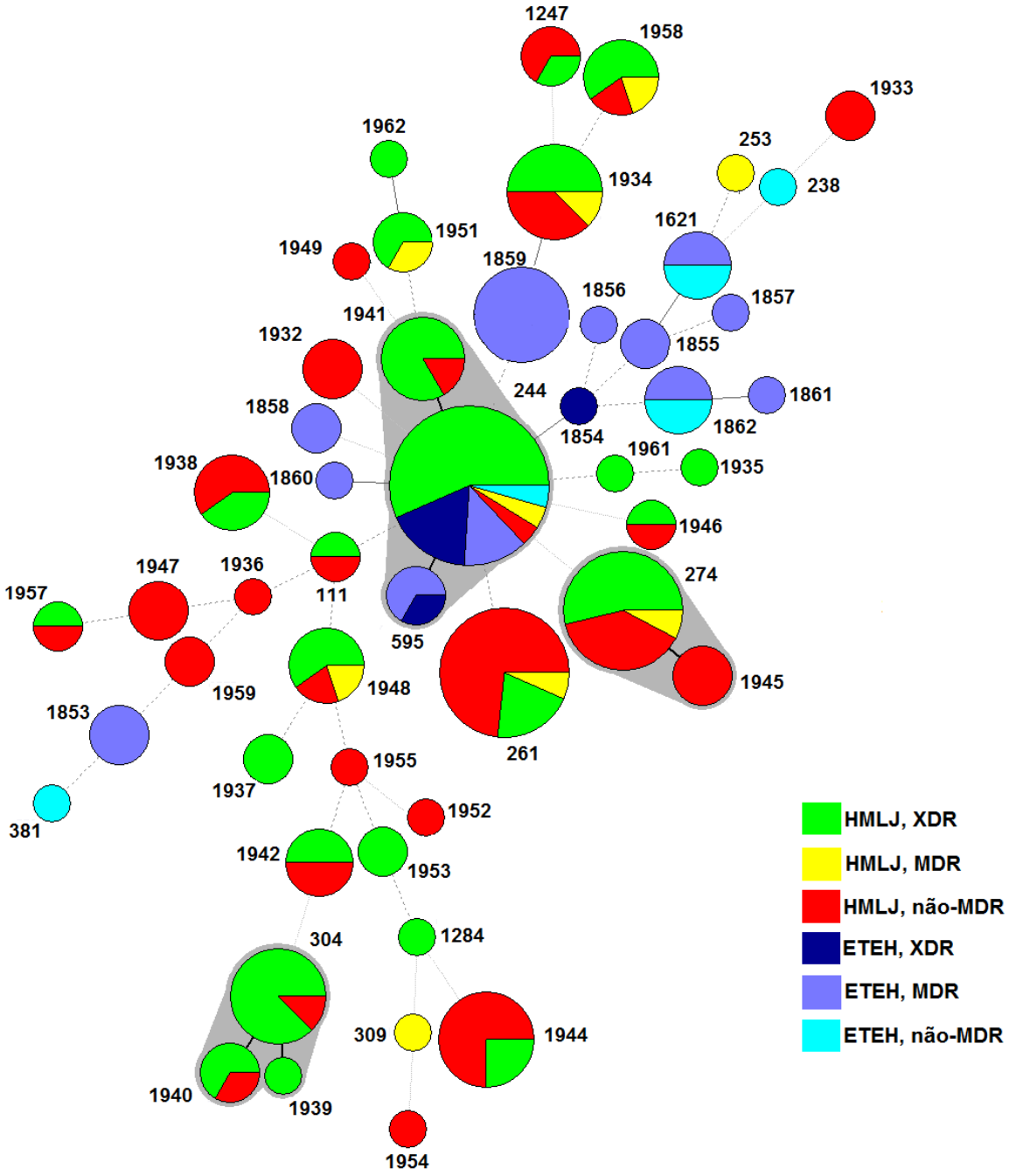
A árvore foi baseada na análise do perfil alélico dos genes *acs*A, *aro*E, *gua*A, *mut*L, *nuo*D, *pps*A and *trp*E. Isolados não Multidroga Resistente (não-MDR), Multidroga Resistente (MDR) e Extensivamente Droga Resistente (XDR) foram indicados ao lado de cada ST. Círculos indicam o ST específico e o número de isolados de acordo com o tamanho do círculo. As cores dos círculos indicam o local de isolamento dos isolados. Novos ST estão marcados com (*). Linhas que ligam os ST, indicam que eles diferem em um alelo (linha grossa sólida), ou 3 a 5 locus (linhas finas e pontilhadas). O halo cinza que circula os ST indica a formação de um complexo clonal. A relação entre os ST foi determinada pela análise do BioNumerics (BioNumerics 6.6) aonde o CC foi formado com ST com único ou duplo locus variante.

5.2.2.3. Análise Comparativa - Isolados da ETEH e HMLJ

A análise comparativa entre os perfis gerados pela ERIC-PCR demonstrou uma maior diversidade nos isolados da ETEH (49%), em relação aos isolados do HMLJ (36%) resultando em um total de 69 perfis nos ambientes estudados. Estes 69 perfis, foram agrupados em 50 ST, pela análise através do MLST.

Os dados gerados a partir dos 50 ST na árvore de MST, demonstraram o agrupamento em 3 CC, sendo dois CC formados apenas com isolados do HMLJ: CC304 (ST304, ST1939 e ST1940) e CC274 (ST274 e ST1945) e um CC com isolados dos dois ambientes estudados: CC244 (ST244, ST595 e ST1941). O ST244 foi o ST com maior número de isolados e o único que esteve presente tanto na ETEH quanto no HMLJ, com maior prevalência nos isolados XDR do HMLJ, XDR da ETEH e MDR da ETEH (Figura 11).

Figura 11. *Minimum spanning tree* (MST) dos ST do MLST de *Pseudomonas aeruginosa* isolados da ETEH e HMLJ



A árvore foi baseada na análise do perfil alélico dos genes *acs*A, *aro*E, *gua*A, *mut*L, *nuo*D, *pps*A and *trp*E. Círculos indicam o ST específico e o número de isolados de acordo com o tamanho do círculo. As cores dos círculos indicam o local de isolamento e o perfil de resistência dos isolados não Multidroga Resistente (não-MDR), Multidroga Resistente (MDR) e Extensivamente Droga Resistente (XDR). Linhas que ligam os ST, indicam que eles diferem em um alelo (linha grossa sólida), ou 3 a 5 locus (linhas finas e pontilhadas). O halo cinza que circula os ST indica a formação de um complexo clonal. A relação entre os ST foi determinada pela análise do BioNumerics (BioNumerics 6.6) aonde o CC foi formado com ST com único ou duplo locus variante.

6. DISCUSSÃO

A distribuição de *P. aeruginosa* multidroga resistentes está associada à sua ampla colonização nos hospitais e em seu efluente, de onde são lançadas aos ambientes aquáticos. Embora os processos de tratamento desses efluentes sejam capazes de reduzir significantemente o número de bactérias, a concentração de microrganismos resistentes no efluente tratado ainda pode ser significativamente elevada (KORZENIEWSKA; HARNISZ, 2013).

P. aeruginosa pode expressar vários mecanismos de resistência, porém, a produção de enzimas beta-lactamases (ESBL e carbapenemase) é um dos principais mecanismos de resistência adquirida aos antibióticos beta-lactâmicos, que leva ao rompimento da ligação amida do anel beta-lactâmico (SYKES; MATTEW, 1976; McGOWAN, 2006).

Este estudo, teve como objetivo principal investigar o perfil de resistência aos antibióticos, principalmente em relação à produção de enzimas de resistência aos beta-lactâmicos e na determinação da relação genética entre os isolados de *P. aeruginosa* de espécimes clínicos e do efluente hospitalar.

Um dado relevante neste estudo, e totalmente oposto a outros relatados na literatura científica, foi a presença de alto percentual de resistência à fosfomicina, tanto nos isolados clínicos (100%) como naqueles do efluente (88%), uma vez que este fármaco tem sido utilizado apenas como alternativa no tratamento de infecções causadas por *P. aeruginosa*, *K. pneumoniae, Escherichia coli*, entre outros organismos MDR produtores de ESBL (PITOUT; LAUPLAND, 2008; DINH et al, 2012). Esse dado é surpreendente pois atualmente a prescrição de fosfomicina é inferior a outras classes de antibióticos, o que poderia provocar uma menor pressão seletiva e consequentemente maior susceptibilidade. Este foi o caso de um estudo envolvendo 4264 pacientes, onde 74,6% apresentaram *Escherichia coli* (76.7%), *Enterococcus faecalis* (4.0%), *Staphylococcus saprophyticus* (3.6%), *Klebsiella pneumoniae* (3.5%) e *Proteus mirabilis* (3.5%) na urina. As cepas de *E. coli* demonstraram as maiores taxas de susceptibilidade à fosfomicina (98.1%) (NABER et al, 2008).

Uma das hipóteses para esse significante aumento da resistência à fosfomicina nos isolados do hospital e do efluente neste estudo, é o possível aumento da prescrição dessa droga no hospital estudado. Sendo assim, a crescente diminuição da susceptibilidade a diversas classes de antibióticos leva a necessidade de reavaliação das escolhas terapêuticas. Até mesmo antibióticos de primeira escolha como os carbapenêmicos começam a ser ineficazes no tratamento de infecções bacterianas. Novos fármacos muitas vezes demoram a serem sintetizados e o recurso a antibióticos antigos e pouco prescritos, como a fosfomicina, pode ser uma alternativa (NARCISO, 2011).

A fosfomicina apresenta um amplo espectro de ação sobre bactérias Gram positivas (*Staphylococcus aureus* e *Streptococcus* spp.), Gram negativas como *P. aeruginosa*, *Neisseria spp.* e espécies da família Enterobacteriaceae, assim como, organismos anaeróbios (POPOVIC et al, 2010). Apresenta baixa toxicidade, não apresenta resistência cruzada com outras classes de antibióticos, mas demonstra sinergismo no tratamento de bactérias Gram negativas e positivas (FOLTZ; WALLICK; ROSENBLUM, 1969; KASTORIS, 2010).

Em *P. aeruginosa*, o transportador parcialmente constitutivo de glicerol-3-fosfato (GlpT) foi descrito como sendo o único transportador da fosfomicina, pois este microrganismo não tem nenhum transporte específico para a glucose -6-fosfato nem possui o gene uhpT que codifica o transportador alternativo da fosfomicina em outros gêneros bacterianos (CASTANEDA-GARCIA et al, 2009). Mutações que ocorrem essencialmente no gene *glp*T estão associadas a uma diminuição da susceptibilidade (TAKAHATA et al, 2010), o que pode justificar o alto percentual de resistência à fosfomicina encontrada neste estudo.

Outro dado igualmente relevante, em relação à susceptibilidade aos antibióticos, foi o baixo percentual de resistência às polimixinas B e colistina, tanto nos isolados clínicos: colistina (8%) e polimixina B (7%), quanto nos isolados do efluente: colistina (17%) e polimixina B (20%). Essas drogas têm um espectro de ação antimicrobiana apenas em bactérias Gram negativas. O Programa de Vigilância Epidemiológica (SENTRY - 2001 a 2004) demonstrou excelente atividade da polimixina B frente a cepas de *P. aeruginosa* (1,3% de resistência) (GALES; JONES; SADER, 2006). No entanto, o principal efeito adverso desta classe de antibióticos é a nefrotoxicidade o que tem sido motivo de várias revisões (EVANS;

FEOLA; RAPP, 1999; FALAGAS; KASIAKOU, 2006). Com isso, as polimixinas deixaram de ser usadas rotineiramente e foram substituídas por outros agentes antimicrobianos com toxicidade inferior (MENDES; BURDMANN, 2009). Na década de 90, com o aparecimento de bactérias multirresistentes, inclusive aos antibióticos beta-lactâmicos, aminoglicosídeos e quinolonas, ressurgiu o interesse pelas polimixinas. Desta forma, surgiram ensaios clínicos randomizados avaliando a eficácia das polimixinas frente as bactérias multirresistentes (ZAVASCKI et al, 2007b).

Os isolados de *P. aeruginosa,* recuperados nos cinco pontos da ETEH, apresentaram altos percentuais de resistência MDR (82%) e XDR (18%), sendo os níveis de susceptibilidade à fosfomicina, tobramicina, levofloxacina, ciprofloxacina e piperacillina/tazobactam menores nas primeiras etapas do tratamento e significantemente maiores ($p<0.05$) nas etapas finais. Essas alterações nos permitem considerar a *P. aeruginosa* um bom marcador na avaliação do impacto do tratamento de efluentes no comportamento do resistoma microbiano. O mesmo foi observado em relação aos isolados clínicos, onde um aumento significativo ($p<0.05$) da resistência foi demonstrada nas diferentes alas do HMLJ em relação à ticarcillina/ácido clavulânico (cirurgia geral), polimixina B e colistina (CTI), ciprofloxacina e levofloxacina (Maternidade) e meropenema e piperacillina/tazobactam (UTI).

É importante destacar que 100% dos isolados da etapa do tratamento de cloração apresentaram perfil XDR. Desde a década de 1970, estudos sobre o efeito da cloração em bactérias resistentes aos antibióticos, descrevem um aumento considerável de bactérias resistentes a múltiplos antibióticos na água e no esgoto (GRABOW et al, 1973; ARMSTRONG et al, 1982; MURRAY et al, 1984). Em um estudo anterior, verificamos ausência de crescimento microbiano no ponto de cloração e presença de bactérias viáveis na etapa seguinte, sugerindo um efeito bacteriostático do cloro sobre as células que se apresentaram viáveis na etapa seguinte (SANTORO; ROMÃO; CLEMENTINO, 2012). Esses resultados podem estar associados também à concentração de cloro dispensado na fase final do tratamento, possibilitando efeitos bactericida e/ou bacteriostático no momento da coleta das amostras. Além disso, outras condições, tais como população microbiana em diferentes estágios de desenvolvimento, concentração de desinfetante, alto fluxo

do efluente e tempo de contato reduzido, podem ter interferido no processo de desinfecção (NWACHCUKU; GERBA, 2004).

Neste estudo, foi demonstrada a presença de genes codificadores de ESBL (*bla*$_{SHV}$, *bla*$_{CTX-M-1}$, *bla*$_{CTX-M-2}$ e *bla*$_{TEM}$), KPC (*bla*$_{KPC}$) e MBL (*bla*$_{VIM}$ e *bla*$_{SPM}$) tanto em isolados clínicos como nos da ETEH, que apresentou percentual superior a todos os genes em relação aos isolados do HMLJ. Vale ressaltar, que as variantes *bla*$_{CTX-M-1}$ e *bla*$_{CTX-M-2}$ foram detectadas exclusivamente nos isolados da ETEH e HMLJ, respectivamente (Tabela 8). Porém, apesar de ter sido detectada no efluente, nós acreditamos que a variante *bla*$_{CTX-M-1}$ é originária do ambiente hospitalar, uma vez que ela foi detectada no primeiro ponto de coleta da ETEH, ou seja, no efluente hospitalar. Além disso, não verificamos relatos a respeito de *P. aeruginosa* portando *bla*$_{CTX-M-1}$ em isolados do efluente hospitalar.

A primeira enzima do grupo CTX-M (CTX-M-1) foi detectada em *E. coli* de origem clínica na Alemanha em 1989 (BAUERNFEIND; CHONG; SCHWEIGHARTl, 1990). Em *P. aeruginosa*, a primeira enzima, CTX-M-1 foi observada em isolados de amostras de escarro de pacientes com fibrose cística em Amsterdam (AL NAIEMI; DUIM; BART, 2006). Posteriormente, foram identificadas substituições aminoacídicas que deram origem a novas variantes da enzima CTX-M (DIAS, 2009).

Há poucos relatos em todo o mundo sobre a estrutura populacional de *P. aeruginosa* produtoras de CTX-M (PICÃO et al, 2009a; POLOTTO et al, 2012). A localização de *bla*$_{CTX-M-2}$ em *P. aeruginosa* acredita-se ser um resultado da sua transferência a partir de Enterobacteriaceae (PICÃO et al, 2009b). Recentemente, um estudo de Tollentino e colaboradores (2011) demonstrou uma alta prevalência de *K. pneumoniae* codificando *bla*$_{CTX-M-2}$ em um mesmo hospital do Brasil. Este pode ter sido o reservatório para a transmissão horizontal (POLOTTO et al, 2012). A identificação destes genes em isolados de *P. aeruginosa* é alarmante e mostra que a mobilização horizontal do gene *bla*$_{CTX-M}$ entre diferentes gêneros e espécies é uma realidade no Brasil (SILVA, 2009).

Um estudo de Galetti e colaboradores (2015), em dois hospitais de regiões distintas do Brasil, também verificou a presença do gene *bla*$_{CTX-M-2}$ em isolados clínicos. Embora o gene *bla*$_{CTX-M}$ tenha sido identificado com menor frequência neste estudo, as beta-lactamases codificadas por ele estão em rápida expansão (TZOUVELEKIS et al, 2000). Atualmente, a família CTX-M é formada por mais de

120 enzimas (JACOBY; BUSH, 2012), que são endêmicas em muitos países, com emergência hospitalar e comunitária (LIVERMORE et al, 2007).

O gene *bla*$_{TEM}$ foi o prevalente tanto nos isolados clínicos (22%) quanto nos da ETEH (45%). Várias beta-lactamases de origem plasmidial são produzidas por patógenos Gram-negativos, porém a variante mais comum é a TEM-1, que tem sido considerada uma enzima muito eficiente (BUSH; JACOBY; MEDEIROS, 1995). Outras quatro variantes do tipo TEM (TEM-4, TEM-21, TEM-24 e TEM-42) também foram descritas em isolados clínicos de *P. aeruginosa* na França (MUGNIER et al, 1996; MARCHANDIN et al, 2000).

Comparando o percentual de detecção dos genes de beta-lactamase, nos dois ambientes estudados, verificamos que entre os isolados da ETEH, o percentual foi maior para todos os genes em comum. Essa observação pode ser explicada pela alta capacidade da *P. aeruginosa* em habitar ambientes aquáticos por longos períodos, interagindo com outros microrganismos presentes no ambiente criando dessa forma, vias de disseminação e reservatórios ambientais de genes de resistência aos antibióticos (PERRON; GONZALEZ; BUCKLING, 2008).

Neste estudo, oito isolados clínicos, apresentaram associados a dois ou três genes *bla*: um isolado XDR do CTI (*bla*$_{KPC}$ e *bla*$_{VIM}$), um isolado XDR do CTI (*bla*$_{CTX-M2}$ e *bla*$_{TEIM}$), um isolado XDR do CTI (*bla*$_{TEM}$ e *bla*$_{KPC}$), três isolados XDR do CTI, pediatria e NI (*bla*$_{CTX-M-2}$, *bla*$_{TEM}$ e *bla*$_{KPC}$), um isolado XDR do CTI (*bla*$_{CTX-M-2}$, *bla*$_{TEM}$ e *bla*$_{VIM}$) e um isolado XDR do local NI (*bla*$_{TEM,}$ *bla*$_{KPC}$ e *bla*$_{VIM}$), com a maioria dos isolados proveniente do CTI. Atualmente, é preocupante o aumento do número de isolados de *Pseudomonas aeruginosa* com genes *bla* principalmente em CTI (LOUREIRO et al, 2002). Os pacientes do CTI são clinicamente graves, geralmente com internações prolongadas e em uso de procedimentos invasivos (cateteres venosos centrais, sondas vesicais de demora e ventilação mecânica), sendo assim mais suscetíveis ao desenvolvimento de infecções especialmente por microrganismos multirresistentes (MARKOGIANNAKIS et al, 2009). Um dos fatores de maior impacto nesse aumento da resistência microbiana é a administração de altas doses de antibióticos em pacientes criticamente enfermos. Além disso, o período permanência no CTI também proporciona um aumento da possibilidade de colonização por cepas resistentes nos pacientes, conforme demonstrado por Tsukayama e colaboradores (2004).

Em todos os pontos de isolamento da ETEH, desde o gradeamento até o efluente tratado, foram detectadas linhagens contendo pelo menos 2 genes *bla* associados. É importante ressaltar que os genes *bla*$_{TEM}$, *bla*$_{KPC}$, *bla*$_{VIM}$ e *bla*$_{SPM}$ foram encontrados em isolados no quinto e último ponto da ETEH, podendo se espalhar por todo o ambiente aquático, possibilitando assim a transmissão dessas cepas para a população e a disseminação de genes de resistência aos antibióticos. Embora o tratamento do esgoto líquido dos hospitais seja capaz de remover até quatro ciclos logarítmicos de bactérias, a prevalência de bactérias multirresistentes normalmente não diminui (GALVIN et al, 2010; VARELA et al, 2014). Em vez disso, assim como neste estudo, alguns perfis de resistência podem até mesmo tornar-se mais prevalente após o processo de tratamento (NOVO et al, 2013).

A descarga de bactérias multirresistentes, incluindo produtores de KPC, nos ambientes aquáticos da cidade, é preocupante, uma vez que esses isolados podem persistir no ambiente e atuar como patógenos oportunistas e/ou reservatórios de resistência que podem acelerar a evolução da resistência antimicrobiana na comunidade (KIM; AGA, 2007; BAQUERO; MARTINEZ; CANTON, 2008; MARTINEZ, 2009).

A presença de *Pseudomonas pseudoalcaligenes* e *P. aeruginosa* carreando o gene *bla*$_{VIM-2}$ em efluente hospitalar e outros ambientes aquáticos sugerem que a propagação contínua destes genes está ocorrendo simultaneamente em diferentes ambientes e pode ocorrer em diferentes espécies bacterianas (QUINTERA; PEIXE, 2006). O gene *bla*$_{SPM-1-MBL}$ é o mais prevalente no Brasil; sua presença foi verificada em isolados de *P. aeruginosa* em amostras do efluente hospitalar e água superficial, em um estudo cujo objetivo foi verificar a disseminação epidemiológica desse gene em amostras ambientais no sul do Brasil (FUENTEFRIA et al, 2008).

A liberação de altas concentrações de antibióticos e genes de resistência em ecossistemas naturais é um evento recente em termos evolutivos. No entanto, esta poluição pode impactar na estrutura e atividade de comunidades microbianas do ambiente. Em corpos d'água, bactérias de diferentes origens (humana, animal e ambiental) são capazes de se misturar, e como consequência desta troca promíscua, a resistência evolui através da transferência horizontal de genes. (BAQUERO; MARTINEZ; CANTON, 2008).

Os 41 isolados da ETEH foram agrupados em 15 ST diferentes, com 5 ST previamente descritos e dez novos. Destes dez, nove foram associados a pelo menos uma cepa MDR e o ST-1854 contém uma cepa XDR. Outros isolados XDR foram incluídos nos ST244 e ST595. Os 136 isolados do HMLJ foram agrupados, pela MLST, em 36 ST diferentes, sendo nove previamente descritos e 27 novos. Entre os novos ST, 4 foram associados a pelo menos uma cepa MDR e uma XDR e 13 a pelo menos uma cepa XDR. Outros isolados XDR foram classificados como ST111, ST244, ST261, ST274, ST304, ST1247, ST1284, Todos os ST, identificados neste estudo, foram depositados na base de dados de MLST para *P. aeruginosa*.

Nossos resultados demonstraram uma alta diversidade associada aos perfis MDR e XDR não só em isolados clínicos, mas pela primeira vez, em isolados provenientes do efluente hospitalar no Brasil. É importante ressaltar que o ST244 se encontra distribuído em todo o mundo com 49 isolados na base de dados do MLST, sendo 10 isolados no Brasil: 9 deste estudo (3 do efluente hospitalar e 6 de amostras clínicas) e uma amostra clínica (MAÂTALLAH et al, 2013; BAE et al, 2014; CHEN et al, 2014). A análise da relação clonal associado aos perfis de resistência também demonstrou alta diversidade genética em isolados clínicos de *P. aeruginosa* multidroga resistentes inclusive aos antibióticos carbapenêmicos na Espanha (GARCÍA-CASTILLO et al, 2011; GOMILA et al, 2013).

Dois isolados pertencentes ao ST595, depositados no banco de dados do MLST, foram isolados no Brasil, a partir de amostras não clínicas, um deste estudo que foi isolado no efluente hospitalar e outro de fonte não divulgada. Três isolados clínicos associados ao ST304 foram deste estudo, sendo que o primeiro isolado que deu origem ao ST304 não está mais depositado na base de dados do MLST (última consulta ao banco em 03/11/2015). O ST 253 está associado ao CC PA14, descrito no banco de dados de *P. aeruginosa*, e está bem distribuído com 114 isolados (solo, água e amostras clínicas), sendo 1 isolado do Brasil, de uma amostra clínica deste estudo.

A associação dos ST para formar complexos clonais (CC) com base no número de alelos compartilhados não é uma regra bem definida e depende da plasticidade e do tamanho do genoma do organismo analisado. O banco de dados de MLST de *P. aeruginosa* descreve apenas dois CC: PA01 associado ao ST549 e PA14 associado ao ST253 e nenhuma informação é divulgada de como esses CC

foram formados já que só há apenas um ST para cada CC. Maâtallah e colaboradores (2013) consideraram que os ST que compartilham cinco ou mais alelos idênticos fazem parte do mesmo CC. Seguindo essa regra, eles descreveram 11 CC com dois grandes clones formados por cinco ST cada, compartilhando 5 ou mais alelos idênticos. Um deles (CC244) nomeado pelo seu ST central (ST244), inclui outros ST geneticamente relacionados (ST990, ST993, ST986 e ST654). Dois ST pertencentes ao CC244 (ST244 e ST654), incluíram vários isolados clínicos com susceptibilidade reduzida a diferentes classes de antibióticos, produzindo ESBL, IMP-type MBL e KPC. Não foi possível comparar as cepas associadas aos ST990, ST986 e ST654, incluídas também no CC244, porque estes ST foram retirados do banco de dados de MLST para *P. aeruginosa*.

O baixo número de depósitos de cepas de *P. aeruginosa* não permite aos seus curadores determinar com segurança novos CC. Alguns estudos relatam a ocorrência de CC específicos para *P. aeruginosa* baseados em seus resultados com um número limitado de ST o que pode levar a interpretações equivocadas. As análises dos nossos dados através de *Minimum Spanning Tree,* confirmaram a presença de alguns dos CC descritos por outros autores além de novos CC. Assim, decidimos seguir a nomenclatura já descrita por esses autores para facilitar as análises das relações entre os isolados dos dois ambientes estudados, alocando novos ST nos CC já descritos ou descrevendo novos CC quando possível.

Neste estudo, os dados gerados a partir da árvore de MST dos ST dos isolados da ETEH, mostraram que o ST595 compartilha seis alelos idênticos com o ST244 e deve ser incluído no CC244. As três cepas classificadas como ST595 foram produtoras de beta-lactamase e apresentaram suscetibilidade reduzida: uma cepa apresentou fenótipo de produtores de KPC e MBL (MDR), outra de produtora de ESBL e MBL (XDR) e a uma terceira ESBL (MDR). Oito isolados classificados como ST244 e também incluídos no CC244 apresentaram padrões de susceptibilidade semelhantes com cepas MDR e XDR, produtoras de KPC, ESBL e MBL. Com esses resultados é possível afirmar que a etapa de cloração desempenhou um papel importante na seleção de isolados MDR e XDR pertencentes ao CC244, onde todos os isolados a partir deste ponto foram agrupados. Até o momento, o CC244 contém isolados de diferentes fontes, como espécimes clínicos, do solo e de água

(CHOLLEY et al, 2014). A partir deste estudo, o CC244 incluiu cepas de *P. aeruginosa* MDR, XDR e não-MDR de isolados do efluente hospitalar.

Os dados gerados a partir da árvore de MST dos ST dos isolados do HMLJ, resultaram na formação de 3 CC: CC244 (ST244 e ST1941) já descrito na literatura, sendo o ST1941 incluído neste estudo e dois CC novos: CC274 (ST274 e ST1945) e CC304 (ST304, ST1939 e ST1940). O CC244 incluiu 15 isolados do ST244 (13 XDR produtoras de ESBL, KPC e MBL e 1 MDR) e 6 do ST1941 (5 XDR produtoras de MBL). O CC274 incluiu 13 isolados do ST274 (8 XDR, produtores de ESBL e KPC) e 3 do ST1945 (que não apresentaram perfil de multirresistência). O CC304 agrupou oito isolados do ST304 (7 apresentaram perfil XDR, sendo 3 produtoras de ESBL), um do ST1939 (XDR produtor de ESBL) e 3 do ST1940 (2 XDR produtores de ESBL, KPC e MBL).

A árvore do MST com todos os 50 ST (HMLJ e ETEH) gerou 3 CC e 42 ST “singletons” não associados a nenhum CC. O principal complexo clonal foi o CC244 formado por 3 ST (244, 595 e 1941) que agrupou 32 isolados (11 da ETEH e 21 do HMLJ) dos 177 estudados. Em relação ao perfil de resistência demonstramos que o CC244 teve o maior número de isolados XDR do HMLJ, seguido de XDR da ETEH e MDR da ETEH. A maioria dos ST “singletons” foram representados por isolados XDR do HMLJ, o contrário do encontrado em estudo recente, onde isolados clínicos de um hospital da Espanha apresentaram 26 ST “singletons” contendo principalmente isolados não-MDR (GOMILA et al, 2013). Essa discrepância pode sinalizar uma maior propagação de organismos e genes de resistência no hospital e em seu efluente, devido principalmente à administração indiscriminada de antibióticos no Brasil.

A análise comparativa da relação clonal e do resistoma de *P. aeruginosa* de origem clínica e do efluente revelou o CC244 contendo cepas MDR e XDR. O potencial patogênico deste clone, presente em várias unidades do hospital, no efluente tratado que é lançado para o corpo hídrico receptor, passa a ter um papel importante na propagação de organismos e genes multirresistentes.

Nossos resultados mostraram que o tratamento dispensado ao efluente hospitalar promoveu um aumento na taxa de resistência aos antibióticos, o que nos leva a sugerir a ocorrência da transferência horizontal de genes de resistência mediada pela pressão seletiva (MARTINEZ, 2009). Estratégias para reduzir esse

quadro são necessárias, incluindo a neutralização de antibióticos nas águas residuais e no meio ambiente (BERGLUND et al, 2014). Vários projetos de pesquisa têm sido realizados com o objetivo de avaliar a adequação de alguns sistemas de tratamento para o efluente hospitalar. Eles referem-se geralmente a uma discussão relacionada à eficiência de remoção de contaminantes convencionais e microrganismos, e as possibilidades de reutilizar esta água recuperada na irrigação para enfrentar problemas decorrentes da escassez de água (CHITNIS et al, 2004; SHRESTHA; HABERL; LABER, 2001).

Em países europeus esforços são realizados para melhorar a remoção de compostos persistentes, onde as tecnologias de processos de oxidação avançada são os mais investigados. Estudos em geral referem-se à presença e à remoção de fármacos, bem como, de avaliações eco toxicológicas (VERLICCHI et al, 2010; ESCHER et al, 2011). Diferentes ETEH já foram construídas para o tratamento de efluente hospitalar, em escala piloto. Cada uma consiste em tratamento preliminar como o biorreator de membrana (BEIER et al, 2011), seguido de ozonização e ultravioleta ozonização e carvão em pó ativado e ozonização e carbono ativado granular (VERLICCHI et al, 2010).

Neste estudo, a genotipagem associada aos perfis de resistência de *P. aeruginosa* do hospital e de seu efluente mostrou-se efetiva para detectar um complexo clonal multirresistente. Essa evidência poderá auxiliar no estabelecimento de medidas para o controle da disseminação de resistência na unidade de saúde e nos ecossistemas aquáticos do Rio de Janeiro.

7. CONCLUSÕES

- Nossos resultados mostraram a presença de *P. aeruginosa* multidroga resistentes em isolados clínicos e nos isolados das diferentes etapas da ETEH, sendo assim considerada um bom marcador no estudo do comportamento do resistoma microbiano;
- A frequência de *P. aeruginosa* com fenótipos MDR e XDR em isolados clínicos e em isolados do efluente sugere altas taxas de transferência horizontal de genes de resistência nesses ambientes;
- ERIC-PCR indicou uma maior variabilidade genética entre os isolados de *P. aeruginosa* do que a observada por MLST, os isolados com perfis idênticos foram considerados o mesmo ST;
- Trinta e quatro por cento dos isolados da ETEH e HMLJ, foram agrupados em 3 Complexos Clonais CC244, CC274 e CC304, sendo o complexo principal CC 244 que incluiu, principalmente, linhagens XDR e MDR dos dois ambientes estudados;
- O ST244 demonstra que isolados multirresistentes do hospital foram liberados para o efluente e que mesmo após o tratamento foram lançados o ambiente aquático disseminando organismos e genes de resistência;
- Nossos resultados apontam a necessidade de uma maior conscientização na administração de antibióticos nas unidades de saúde e da implementação de tratamentos mais eficientes na remoção de organismos multirresistentes com o objetivo de preservar o meio ambiente e a saúde da população.

REFERÊNCIAS

ABDEL-GAWAD, H. A.; ALY, A. M. Assessment of aquatic environmental for wastewater management quality in the hospitals: a case study. **Aust J Basic Appl Sci**, v.5, p.474–482, 2011.

ABESSA, D. M. S. **Avaliação da Qualidade de Sedimentos do Sistema Estuarino de Santos, SP, Brasil.** 2002. 290f. Tese (Doutorado em Oceanografia) - Instituto. **O Panorama dos Resíduos Sólidos no Brasil 2013**. 11ª edição do relatório anual lançado em 04 de agosto de 2014.

ABRELPE – Associação Brasileira de Empresas de Limpeza Pública e Resíduos Especiais **Reserch**, v. 36, p. 697-705, 2013.

AHMED, O. I. et al. Detection of *bla*$_{SHV}$ and *bla*$_{CTX-M}$ genes in ESBL producing *Klebsiella pneumoniae* isolated from Egyptian patients with suspected nosocomial infections. **The Egyptian Journal of Medical Human Genetics**, v. 14, p. 277–283, 2013.

AL-AHMAD, A.; DASHNER, F. D.; KÜMMERER, K. Biodegradability of Cefotiam, Ciprofloxacin, Meropenem, Penicilin G and Sulfametazole and Inhibition of Waste Water Bacteria. **Arch Env Contam Toxicol**, v. 37, p. 158-63, 1999.

AL NAIEMI, N.; DUIM, B.; BART, A. A CTX-M extended-spectrum β-lactamase in *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia*. **J Med Microbiol**, v. 55, p. 1607–1608, 2006.

AMBLER, R. P. The structure of beta-lactamases. **Philos Trans R Soc Lond B Biol Sci**, v. 289, p. 321-331, 1980.

ARMSTRONG, J. L.; CALOMIRIS, J. J.; SEIDLER, R. J. Selection of antibiotic-resistant standard plate count bacteria during water treatment. **Appl Environ Microbiol**, v. 44, p. 308–316, 1982.

BABIC, M.; HUJER, A. M.; BONOMO, R. A. What is new in antibiotic resistance? Focus on Beta-lactamases. **Drug Resistance Updates**, v. 9, p.142-156, 2006.

BAE, I. K. et al. Molecular epidemiology of *Pseudomonas aeruginosa* clinical isolates from Korea producing β-lactamases with extended-spectrum activity. **Diagn Microbiol Infect Dis**, v. 79, p. 373-377, 2014.

BAHMANI, N.; RAMAZANZADEH, R. Detection of SHV type extended-spectrum B-lactamase and risk factors in *Pseudomonas aeruginosa* clinical isolates. **Pak J Med Sci**, vol. 29, p. 788-792, 2013.

BANNING, N.; TOZE, S.; MEE, B. J. Persistence of biofilm-associated *Escherichia coli* and *Pseudomonas aeruginosa* in groundwater and treated effluent in a laboratory model system. **Microbiology**, v. 149, p. 47-55, 2003.

BAQUERO, F.; MARTINEZ, J. L.; CANTON, R. Antibiotics and antibiotic resistance in water environments. **Curr Opin Biotechnol**, v. 19, p. 260–265, 2008.

BARCELLOS, C.; QUITÉRIO, L. A. D. Vigilância ambiental em saúde e sua implantação no Sistema Único de Saúde. **Revista de Saúde Pública**, v. 40, p.170-77, 2006.

BARGUIGA, A. et al. Prevalence and genotypic analysis of plasmid-mediated β-lactamases amongurinary *Klebsiella pneumoniae* isolates in Moroccan community. **J Antibiot**, v. 66, p. 11-16, 2013.

BAUERNFEIND, A.; CHONG, Y.; SCHWEIGHART, S. Extended broad spectrum beta-lactamase in *Klebsiella pneumoniae* including resistance to cephamycins. **Infection**, v. 17, p. 316-321, 1990.

BEIER, S. et al. Full scale membrane bioreactor treatment of hospital wastewater as forerunner for hotspot wastewater treatment solutions in high density urban areas. **Water Sci Technol**, v. 63, p. 66–71, 2011.

BERGEY'S manual of systematic bacteriology. 2 ed. New York: Springer, 2011.

BERGLUND, B. et al. Efficient removal of antibiotics in surface-flow constructed wetlands, with no observed impact on antibiotic resistance genes. **Sci Total Environ**, v. 1, p. 476–477, 2014.

BEYENE, H.; REDAIE, G. Assessment of waste stabilization ponds for the treatment of hospital wastewater: the case of Hawassa University Referral Hospital. **World Appl Sci J**, v. 15, p. 142–150, 2011.

BRASIL. Lei nº 8.080, DE 19 de setembro de 1990. Dispõe sobre as condições para a promoção, proteção e recuperação da saúde, a organização e o funcionamento dos serviços correspondentes, e dá outras providências. **Diário Oficial da República Federativa do Brasil**, Brasília, DF, 20 set.1990. Disponível em http://portal.saude.gov.br/portal/arquivos/pdf/lei8080.pdf. Acesso em: 8 nov. 2014.

BRASIL. Fundação Nacional de Saúde. **Vigilância Ambiental em Saúde/Fundação Nacional de Saúde**.- Brasília: FUNASA, 2002.

BRASIL. Agência Nacional de Vigilância Sanitária – ANVISA – RDC n. 306, de 7 de dezembro de 2004. Dispõe sobre o regulamento técnico para o gerenciamento de resíduos de serviços de saúde. **Diário Oficial da União**, Brasília, DF, 10 dez. 2004.

BRASIL. Conselho Nacional do Meio Ambiente - CONAMA – Resolução n° 357, de 17 de março de 2005. Dispõe sobre a classificação dos corpos de água e diretrizes ambientais para o seu enquadramento, bem como estabelece as condições e padrões de lançamento de efluentes, e dá outras providências. **Diário Oficial da União**, Brasília, DF, 18 março de 2005.

BRASIL. Agência Nacional de Vigilância Sanitária - ANVISA. **Manual de gerenciamento de resíduos de serviços de saúde**. Brasília, 2006. 12 p. (Série A. Normas e Manuais Técnicos).

BRASIL. Decreto nº 7.335, de 19 de outubro de 2010. Aprova o estatuto e o quadro demonstrativo dos cargos em comissão e das funções gratificadas da Fundação Nacional de Saúde - FUNASA, e dá outras providências. **Diário Oficial da União**, Brasília, DF, 20 de outubro de 2010a.

BRASIL. Agência Nacional de Vigilância Sanitária – ANVISA - Resolução nº 44 de 28 de outubro de 2010. Dispõe sobre o controle de medicamentos à base de substâncias classificadas como antimicrobianos, de uso sob prescrição médica, isoladas ou em associação e dá outras providências. **Diário Oficial da União**, Brasília, DF, 28 de outubro de 2010b.

BRASIL. Agência Nacional de Vigilância Sanitária – ANVISA - Resolução n° 20 de 5 de maio de 2011. Dispõe sobre o controle de medicamentos à base de substâncias classificadas como antimicrobianos, de uso sob prescrição, isoladas ou em associação. **Diário Oficial da União**, Brasília, DF, 17 de junho de 2011.

BRASIL. Agência Nacional de Vigilância Sanitária – ANVISA - Nota técnica 01/2013. Medidas de prevenção e controle de infecções por enterobactérias multirresistentes. **Ministério da Saúde**, Brasília, DF, 17 de abril de 2013.

BUENO, E. **À sua Saúde – A Vigilância Sanitária na História do Brasil**. Ed. Anvisa. 1ª Edição, 2005. Cadastro Nacional de Estabelecimentos de Saúde-CNES/MS, 2005.

BUSH, K.; JACOBY, G. A.; MEDEIROS, A. A. A functional classification scheme for β-lactamases and its correlation with molecular structure. **Antimicrob Agents Chemother**, v. 39, p. 1211-1233, 1995.

BUSH, K.; JACOBY, G. A. Updated functional classification of beta-lactamases. **Antimicrob Agents Chemother**, v. 54, p. 969-976, 2010.

BUSH, K; JACOBY, G. A. Amino acid sequences for TEM, SHV and OXA extended-spectrum and inhibitor resistant Beta-lactamases. **Lahey Clinic** Disponível em: http://www.lahey.org/studies. Acesso: 21 jul. 2015.

BUSTOS, M.R.L. **A Educação Ambiental sob a Ótica da Gestão de Recursos Hídricos**. 2003. 194f. Tese (Doutorado em Engenharia) - Escola Politécnica da Universidade de São Paulo, 2003.

CÂMARA, V. M.; TAMBELLINI, A. T. Considerações sobre o uso da epidemiologia nos estudos em saúde ambiental. **Rev Bras Epidemiol**, vol. 6, p. 95-104, 2003.

CARVALHO, A. P. et al. Characterization of an epidemic carbapenem-resistant *Pseudomonas aeruginosa* producing SPM-1 metallo-beta-lactamase in a hospital located in Rio de Janeiro, Brazil. **Microb Drug Resist Summer**, v. 12, p. 103-108, 2006.

CASTANEDA-GARCIA, A. et al. The glycerol-3-phosphate permease GlpT is the only fosfomycin transporter in *Pseudomonas aeruginosa*. **J Bacteriol**, v. 191, p. 6968-6974, 2009.

CASTANHEIRA, M. et al. Emergence of the extended spectrum beta-lactamase GES-1 in a *Pseudomonas aeruginosa* strain from Brazil: Report from the SENTRY antimicrobial surveillance program. **Antimicrob Agents Chemother**, v. 48, p. 2344-2345, 2004.

CEZÁRIO, R.C. et al. Infection and colonization by Gram-negative bacilli in neonates hospitalized in high risk nursey at Uberaba Federal University Hospital: etiology, resistant phenotypes and risk factors. **Braz J Microbiol**, v. 35, p. 193-198, 2004.

CHEN, Y. et al. Dissemination of IMP-6-producing *Pseudomonas aeruginosa* ST244 in multiple cities in China. **Eur J Clin Microbiol Infect Dis**, v. 33, p. 1181-1187, 2014.

CHITNIS, V. et al. Bacterial population changes in hospital effluent treatment plant in central India. **Water Res**, v. 38, p. 441–447, 2004.

CHOLLEY, P. et al. Population structure of clinical *Pseudomonas aeruginosa* from West and Central African countries. **PLoS ONE**, v. 4, p. e107008, 2014.

CLAEYS, G. et al. PER-1 beta-lactamase producing *Pseudomonas aeruginosa* in an Intensive care unit. **J Antimicrob Chemother**, v. 45, p. 924-925, 2000.

CLSI – Clinical and Laboratory Standards Institute - **Performance Standards for Antimicrobial Susceptibility Testing** – Twenty-second Informational Supplement.M100-S22, vol. 32, nº 3, 2014.

CORNAGLIA, G.; GIAMARELLOU, H.; ROSSOLINI, G. M. Metallo-β-lactamases: the last frontier for β-lactam? **Lancet Infect Dis**, v. 11, p. 381-393, 2011.

CORVEC, S. et al. AmpC cephalosporinase hyperproduction in *Acinetobacter baumannii* clinical strains. **J Antimicrob Chemother**, v. 52, p. 629–635, 2003.

COSTA, E. A. Vigilância Sanitária, Saúde e Cidadania. Belo Horizonte, MG: Núcleo de Saúde Coletiva, **FAMED/UFMG**, p. 13-27, 2000.

COSTA, M. M. et al. Caracterização epidemiológica, molecular e perfil de resistência aos antimicrobianos de *Escherichia coli* isoladas de criatórios suínos do sul do Brasil. **Pesq Vet Bras**, v. 26, p. 5-8, 2006.

CURRAN, B. et al. Development of a Multilocus Sequence Typing Scheme for the Opportunistic Pathogen *Pseudomonas aeruginosa*. **J Clin Microbiol**, v. 42, p. 5644-5649, 2004.

DALLARI, S. G. A vigilância sanitária no contexto constitucional e legal brasileiro. **Cadernos de Saúde Pública**, Belo Horizonte, v. 4, p. 39-52, 2001.

DALMARCO, E. M.; BLATT, S. L.; CÓRDOVA, C. M. M. Identificação laboratorial de β-lactamases de espectro estendido (ESBLs). **RBAC**, v. 38, p. 171-177, 2006.

De ABREU, P. M. et al. Persistence of microbial communities including *Pseudomonas aeruginosa* in a hospital environment: a potential health hazard. **BMC Microbiol**, v. 8, p. 118, 2014.

De VOS, D. et al. Analysis of epidemic *Pseudomonas aeruginosa* isolates by isoelectric focusing of pyoverdine and RAPD-PCR: modern tools for an integrated anti-nosocomial infection strategy in burn wound centers. **Burns**, v. 23, p. 379-386, 1997.

DHILLON, R. H. P.; CLARK, J. ESBLs: A Clear and Present Danger? **Critical Care Research and Practice**, v. 2012, p. 1-11, 2012.

DIÁRIO OFICIAL DO MUNICÌPIO DO RIO DE JANEIRO. **Programa Especial e Permanente de Fiscalização do Sistema de Tratamento de Esgotos de Hospitais e Clínicas criado pelo Decreto no. 19.789/2001**. Decreto n. 21.006/02. D.O. Ano XV, n. 210, 23 de janeiro de 2002.

DIAS, D. J. **Estudo dos principais mecanismos de resistência aos antibióticos β-lactâmicos em bactérias patogênicas de Gram negativo**. 2009. 100f. Dissertação (Mestrado em Genética Molecular e Biomedicina) - Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, 2009.

DIENSTMANN, R. et al. Avaliação fenotípica da enzima *Klebsiella pneumoniae* carbapenemase (KPC) em Enterobacteriaceae de ambiente hospitalar. **J Bras Patol Med Lab**, v. 46, p. 23-27, 2010.

DINH, A. et al. Fosfomycin: efficacy against infections caused by multidrug-resistant bacteria. **Scand J Infect Dis**, v. 44, p. 182-9, 2012.

DODD, M. C. Potential impacts of disinfection processes on elimination and deactivation of antibiotic resistance genes during water and wastewater treatment. **Journal of Environmental Monitoring**, v. 14, p. 1754-1771, 2012.

DOYLE, D. et al. Laboratory detection of Enterobacteriaceae that produce carbapenemases. **J Clin Microbiol**, v. 50, p. 3877-3880, 2012.

DUBOIS, V. et al. Nosocomial outbreak due to a multiresistant strain of *Pseudomonas aeruginosa* P12: efficacy of Cefepime – Amikacin therapy and analysis of β-lactam resistance. **J Clin Microbiol**, v. 39, p. 2072-2078, 2001.

DUBOIS, V. et al. Prolonged outbreak of infection due to TEM-21-producing strains of *Pseudomonas aeruginosa* and enterobacteria in a nursing home. **J Clin Microbiol**, v. 43, p. 4129-4138, 2005.

EMMANUEL, E. et al. Ecotoxicological risk assessment of hospital wastewater: a proposed framework for raw effluents discharging into urban sewer network. **J Hazardous Mat**, v. A117, p. 1-11, 2005.

ESCHER, B. I. et al. Environmental toxicology and risk assessment of pharmaceuticals from hospital wastewater. **Water Res**, v. 45, p. 75-92, 2011.

EVANS, M. E.; FEOLA, D. J.; RAPP, R. P. Polymyxin B sulfate and colistin: old antibiotics for emerging multiresistant Gram-negative bacteria. **Ann Pharmacother**, v. 33, p. 960-967, 1999.

FALAGAS, M. E.; KASIAKOU, S. K. Toxicity of polymyxins: a systematic review of the evidence from old and recent studies. **Crit Care**, v. 10, R27, 2006.

FOLTZ, E. L.; WALLICK, H.; ROSENBLUM, C. Pharmacodynamics of phosphomycin after oral administration in man. **Antimicrob Agents Chemother**, v. 9, p. 322-326, 1969.

FOXMAN, B. et al. Choosing an appropriate bacterial typing technique for epidemiologic studies. **Epidemio Perspec & Innov**, v. 2, p.1-8, 2005.

FUENTEFRIA, D. B. et al. *Pseudomonas aeruginosa*: disseminação de resistência antimicrobiana em efluente hospitalar e água superficial. **Revista da Sociedade Brasileira de Medicina Tropical**, v. 41, p. 470-473, 2008.

FUENTEFRIA, D. B.; FERREIRA, A. E.; CORÇÃO, G. Antibiotic-resistant *Pseudomonas aeruginosa* from hospital wastewater and superficial water: Are they genetically related? **Journal of Environmental Management**, v. 92, p. 250-255, 2011.

GALES, A. C.; REIS, A. O.; JONES, R. N. Contemporary assessment of antimicrobial susceptibility testing methods for polymixyn B and colistin: review of available interpretative criteria and quality control guidelines. **Journal of Clinical Microbiology**, v. 39, p. 183-190, 2001.

GALES, A. C. et al. Emergence of an IMP-like metallo-enzyme in an *Acinetobacter baumannii* clinical strain from a Brazilian teaching hospital. **Diagn Microbiol Infect Dis**, v. 45, p. 77-79, 2003.

GALES, A. C.; JONES, R. N.; SADER, H. S. Global assessment of the antimicrobial activity of polymyxin B against 54 731 clinical isolates of gram-negative bacilli: report from the SENTRY antimicrobial surveillance program (2001-2004). **Clin Microbiol Infect**, v. 12, p. 315-321, 2006.

GALETTI, R.; ANDRADE, L. N.; DARINI, A. L. C. *Pseudomonas aeruginosa* carrying blaCTX-M-2 in Brazil: The occurrence of 'high-risk clones'? **Journal of Global Antimicrobial Resistance**, v. 3, p. 153-154, 2015.

GALVIN, S. et al. Enumeration and characterization of antimicrobial-resistant *Escherichia coli* bacteria in effluent from municipal, hospital, and secondary treatment facility sources. **Appl Environ Microbiol**, v. 76, p. 4772–4779, 2010.

GARCÍA-CASTILLO, M. et al. Wide dispersion of ST175 clone despite high genetic diversity of carbapenem-no susceptible *Pseudomonas aeruginosa* clinical strains in 16 Spanish hospitals. **J Clin Microbiol**, v. 49, p. 2905–2910, 2011.

GOMES, M. J. P. **Gênero *Pseudomonas* spp**. Microbiologia Clínica - LABACVET - Bacteriologia, 2008.

GOMILA, M. et al. Genetic diversity of clinical *Pseudomonas aeruginosa* isolates in a public hospital in Spain. **BMC Microbiol**, v. 13, p. 138, 2013.

GRABOW, W. O. K.; MIDDENDORFF, I. G.; PROZESKV, O. W. Survival in maturation ponds of coliform bacteria with transferable drug resistance. **Water Res**, v. 7, p. 1589–1597, 1973.

GUSATTI, C. Z. et al. Resistência a β-lactâmicos em *Acinetobacter* spp. isolados de efluente hospitalar no sul do Brasil. **Revista da Sociedade Brasileira de Medicina Tropical**, v. 42, p. 187-189, 2009.

INACIO, H. S. et al. Phenotypic and genotypic diversity of multidrug-resistant *Pseudomonas aeruginosa* isolates from bloodstream infections recovered in the Hospitals of Belo Horizonte, Brazil. **Chemotherapy**, v. 60, p. 54-62, 2014.

INSTITUTO NACIONAL DE CONTROLE DE QUALIDADE EM SAÚDE. **POP 65.3230.001**: Produção e preservação pelo método de liofilização de bactérias da Coleção de Culturas. Rev. 4. Rio de Janeiro, 2015. 12 p. (Manual da Qualidade. Seção 4.3).

INSTITUTO TRATA BRASIL - **Ranking do Saneamento 2014**. São Paulo. Disponível em http://www.tratabrasil.org.br/datafiles/estudos/ranking/relatorio-completo-2014.pdf. Acesso em: 9 nov. 2014.

IVAMA, A. M.; MELCHIOR, S. C. **Vigilância Sanitária: instrumento de promoção e proteção da saúde. Bases da Saúde Coletiva**. 2 ed. Londrina: EDUEL; ABRASCO, 2007.

JACOBY G. A.; BUSH, K. β-lactamase classification and amino acid sequences for TEM, SHV and OXA extended-spectrum and inhibitor resistant enzymes. Burlington, 2012. Disponível em: <http://www.lahey.org/Studies/>. Acesso em: 16 out. 2015.

JÁCOME, P. R. et al. First Report of KPC-Producing *Pseudomonas aeruginosa* in Brazil. **Antimicrob Agents Chemother**, v. 56, p. 4990, 2012.

JASKULSKI, M. R. **Avaliação da presença de ESBL, carbapenemase do tipo KPC e porinas como mecanismo de resistência em cepas de *Klebsiella pneumoniae* e *Enterobacter* spp.** 2013. 131 f. Teses (Doutorado em Medicina e Ciências da Saúde) – Faculdade de Medicina, Pontifícia Universidade Católica do Rio Grande do Sul, 2013.

JOHNSON, A. P.; WOODFORD, N. Global spread of antibiotic resistance: the example of New Delhi metallo-β-lactamase (NDM) - mediated carbapenem resistance. **J Med Microbiol**, v. 62, p. 499-513, 2013.

KASTORIS, A. C. Synergy of fosfomycin with other antibiotics for Gram -positive and Gram-negative bacteria. **Eur J Clin Pharmacol**, v. 66, p. 359-368, 2010.

KIM, S.; AGA, D. S. Potential ecological and human health impacts of antibiotics and antibiotic-resistant bacteria from wastewater treatment plants. **J Toxicol Environ Health B Crit Rev**, v. 10, p. 559–573, 2007.

KISKA, O. L.; GILLIGAN, P. H. ***Pseudomonas***. In: P. R. Murray, E. J. Baron, M. A. Pfaller, F. C. Tenover, and R. H. Yolken (ed.), Manual of clinical microbiology, 7th ed. ASM Press, Washington, D.C., p. 516-526, 1999.

KORZENIEWSKA, E.; HARNISZ, M. Beta-lactamase producing Enterobacteriaceae in hospital effluents. **J Environ Manage**, v. 123, p. 1–7, 2013.

KLOCKGETHER, J. et al. *Pseudomonas aeruginosa* Genomic Structure and Diversity. **Front Microbiol**, v. 2, p. 150, 2011.

LATOUR, X.; LEMANCEAU, P. Carbon and energy metabolism of oxidase-positive saprophytic fluorescent *Pseudomonas spp*. **Agronomie**, v. 17, p. 427-443, 1997.

LAURETTI, L. et al. Cloning and characterization of bla_{VIM}, a new integron-borne metallo-β-lactamase gene from *Pseudomonas aeruginosa* clinical isolate. **Antimicrob Agents Chemother**, v. 43, p.1584-1590, 1999.

LIVERMORE, D. M. Of *Pseudomonas*, porins, pumps and carbapenems. **J Antimicrob Chemother**, v. 47, p. 247-250, 2001.

LIVERMORE, D. M.; WOODFORD, N. The beta-lactamase threat in Enterobacteriaceae, Pseudomonas and *Acinetobacter*. **Trends Microbiol**, v. 14, p. 413-420, 2006.

LIVERMORE, D. M. et al. CTX-M: changing the face of ESBLs in Europe. **J Antimicrob Chemother**, v. 59, p. 165-174, 2007.

LORIAN, V. **Antibiotics in Laboratory Medicine**, fifth ed. Williams & Wilkins, Baltimore, Hong Kong, London, Munich, 2005.

LOUREIRO, M. M. et al. *Pseudomonas aeruginosa*: Study of antibiotic resistance and molecular typing in hospital infection cases in a neonatal intensive care unit from Rio de Janeiro city, Brazil. **Memórias do Instituto Oswaldo Cruz**, v. 97, p. 387-394, 2002.

MAÂTALLAH, M. et al. Four genotyping schemes for phylogenetic analysis of *Pseudomonas aeruginosa*: comparison of their congruence with multi-locus sequence typing. **PLoS ONE**, v. 8, p. e82069, 2013.

MAGIORAKOS, A. P. et al. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. **Clin Microbiol Infect**, v. 18, p. 268-281, 2012.

MAIDEN, M. C. J. et al. Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. **Proc. Natl. Acad. Sci**, v. 95, p. 3140–3145, 1998.

MARCHANDIN, H. et al. Production of a TEM-24 plasmid-mediated extended-spectrum beta-lactamase by a clinical isolate of *Pseudomonas aeruginosa*. **Antimicrob Agents Chemother**, v. 44, p. 213-216, 2000.

MARTINEZ, J. L. Environmental pollution by antibiotics and by antibiotic resistance determinants. **Environ Pollut**, v. 157, p. 2893–2902, 2009.

MARKOGIANNAKIS, H. et al. Infections in a surgical intensive care unit of a university hospital in Greece. **Int J Infect Agents**, v. 13, p. 145-153, 2009.

MASEDA, G. E. et al. Update on catheter related bloodstream infections in ICU patients. **Enferm Infecc Microbiol Clin Suppl**, v. 4, p. 10-15, 2011.

McGOWAN, J. E. Resistance in Nonfermenting Gram-Negative Bacteria: Multidrug Resistance to the Maximum. **The American J of Medicine**, v, 119, p. S29–S36, 2006.

McQUILLIAN, D. et al. Drug Residues in Ambient Water: Initial Surveillance in New Mexico, USA. In: **Annual New Mexico Environmental Health Conference**, Albuquerque, New Mexico, Anais. New Mexico, v. 7, p. 28-30, 2002.

MENDES, R. E. et al. Integron carrying a novel metallo ßLactamase gene, blaIMP-16, and a fused form of aminoglycoside-resistant gene aac (6) -30/aac (6) -Ib: Report from the SENTRY Antimicrobial Surveillance Program. **Antimicrob Agents Chemother**, v. 48, p. 4693-4702, 2004.

MENDES, R. E. et al. Metallo- beta-lactamase. **J Bras Patol Med Lab**, v. 42, p. 103-113, 2006.

MENDES, C. A. C.; BURDMANN, E. A. Polymyxins: review with emphasis on nephrotoxicity. **Rev Assoc Med Bras**, v. 55, p. 752-759, 2009.

MESA, R. J. et al. Extended-spectrum-lactamase-producing Enterobacteriaceae in different environments (humans, food, animal farms and sewage). **Journal of Antimicrobial Chemotherapy**, v. 58, p. 211–215, 2006.

MIGULA, W. Über ein neues System der Bakterien. **Arbeiten aus dem Bakteriologischen Institut der Technischen Hochschule zu Karlsruhe**, v. 1, p. 235-238, 1894.

MONTEIRO, J. et al. First report f KPC-2-producing *Klebsiella pneumoniae* strains in Brazil. **Antimicrob Agents Chemother**, v. 53, p. 333-334, 2009.

MONTEIRO, J. et al. Rapid detection of carbapenemase genes by multiplex real-time PCR. **J Antimicrob Chemother**, v. 67, p. 906-909, 2012.

MUGNIER, P. et al. A TEM-derived extended-spectrum beta-lactamase in *Pseudomonas aeruginosa*. **Antimicrob Agents Chemother**, v. 40, p. 2488-2493, 1996.

MURRAY, G. E. et al. Effect of chlorination on antibiotic resistance profiles of sewage-related bacteria. **Appl Environ Microbiol**, v. 48, p. 73–77, 1984.

NABER, K. G. et al. Surveillance study in Europe and Brazil on clinical aspects and Antimicrobial Resistance Epidemiology in Females with Cystitis (ARESC): implications for empiric therapy. **Eur Urol**, v. 54, p. 1164–1175, 2008.

NARCISO, A. R. C. **Mecanismos de Resistência à Fosfomicina em Bactérias Uropatogênicas**. 2011. 47f. (Mestrado em Microbiologia Aplicada) – Faculdade de Ciências, Universidade de Lisboa, 2011.

NEIDELL, M. J. et al. Costs of healthcare- and community-associated infections with antimicrobial-resistant versus antimicrobial-susceptible organisms. **Clin Infect Dis**, v. 55, p. 807-815, 2012.

NEVES, P. R. *Pseudomonas aeruginosa* multirresistente: um problema endêmico no Brasil. **J Bras Patol Med Lab**, v. 47, p. 409-420, 2011.

NOVO, A. et al. Antibiotic resistance, antimicrobial residues and bacterial community composition in urban wastewater. **Water Res**, v. 47, p. 1875–1887, 2013.

NORDMANN, P. et al. Characterization of a novel extended-spectrum b-lactamase from *Pseudomonas aeruginosa*. **Antimicrob Agents Chemother**, v. 37, p. 962-969, 1993.

NORDMANN, P.; CUZON, G.; NAAS, T. The real threat of *Klebsiella pneumonia* carbapenemase-producing bacteria. **Lancet Infect Dis**, v. 9, p. 228-236, 2009.

NORDMANN, P. et al. Superbugs in the coming new decade; multidrug resistance and prospects for treatment of *Staphylococcus aureus*, *Enterococcus spp*. and *Pseudomonas aeruginosa* in 2010. **Curr Opin Microbiol**, v. 10, p. 436-440, 2010.

NORDMANN, P.; POIREL, L.; DORTET, L. Rapid detection of carbapenemase-producing Enterobacteriaceae. **Emerg Infect Dis**, v. 18, p. 1503-1507, 2012.

NOVAES, R. M. P. **Avaliação da Eficiência de uma Estação de Tratamento de Efluente Hospitalar através da Detecção e Caracterização Molecular de *Pseudomonas aeruginosa* na Cidade do Rio de Janeiro**. 2008. 51f. (Especialização em Vigilância Sanitária) – Instituto Nacional de Controle de Qualidade em Saúde, Fundação Oswaldo Cruz, 2008.

NWACHCUKU, N.; GERBA, C. P. Emerging waterborne pathogens: can we kill them all? **Curr Opin Biotechnol**, v. 15, p. 175-180, 2004.

OTTO, T. D. et al. ChromaPipe: a pipeline for analysis, quality control and management for a DNA sequencing facility. **Genet Mol Res**, v. 7, p. 861-871, 2008.

PALLERONI, N. J. **Introduction to the Family Pseudomonadaceae** In: W. BALOWS, W.; TRÜPER, H. G.; DWORKIN, M.; HARDER, W.; SCHLEIFER, K. H. (ed.), The Prokaryotes, Springer-Verlag, New York, p. 3071-3085, 1992.

PALLERONI, N. J. **Genus I. *Pseudomonas***. In: BRENNER, D. J.; KRIEG, N. R.; STALEY, J. T. (Eds.), Bergey's Manual of Systematic Bacteriology. Part B: The Proteobacteria. Springer, New York, p. 323–379, 2005.

PASTERAN, F. et al. Emergence of NDM-1-producing *Klebsiella pneumoniae* in Guatemala. **J Antimicrob Chemother**, v. 67, p. 1795-1797, 2012.

PEIRANO, G. et al. Carbapenem-hydrolysis β-lactamase KPC-2 in *Klebsiella pneumoniae* isolated in Rio de Janeiro, Brazil. **J Antimicrob Chemother**, v. 63, p. 265-268, 2009.

PEIX, A.; RAMÍREZ-BAHENA, M. H.; VELÁZQUEZ, E. Historical evolution and current status of the taxonomy of genus *Pseudomonas***. Infect Genet Evol**, v. 9, p. 1132-1147, 2009.

PERRON, G. G.; GONZALEZ, A.; BUCKLING, A. The rate of environmental change drives adaptation to an antibiotic sink. **J Evol Biol**, v. 2, p. 1724-1731, 2008.

PICÃO, R. C.; GALES, A. C. ß-Lactamases de Espectro Ampliado (ESBL) em *Pseudomonas aeruginosa*: Pesadelo ou só Imaginação? In: **Prática Hospitalar**, Ano IX, Nº 49, p.79-84, 2007.

PICÃO, R.C. et al. Diversity of β-Lactamases Produced by Ceftazidime-Resistant *Pseudomonas aeruginosa* Isolates Causing Bloodstream Infections in Brazil. **Antimicrob Agents Chemother**, v. 53, p. 3908–3913, 2009a.

PICÃO, R.C. et al. Further identification of CTX-M-2 extended-spectrum beta-lactamase in *Pseudomonas* aeruginosa. **Antimicrob Agents Chemother**, v. 53, p. 2225–2226, 2009b.

PICÃO, R. C. et al. The route of antimicrobial resistance from the hospital effluent to the environment: focus on the occurrence of KPC-producing *Aeromonas* spp. and Enterobacteriaceae in sewage. **Diagn Microbiol Infect Dis**, v. 76, p. 80-85, 2013.

PITOUT, J. D. D. et al. Emergence of Enterobacteriaceae producing extended-spectrum β-lactamases (ESBLs) in the community. **Journal of Antimicrobial Chemotherapy**, v. 56, p. 52-59, 2005.

PITOUT, J. D.; LAUPLAND, K. B. Extended-spectrum beta-lactamase-producing Enterobacteriaceae: an emerging public-health concern. **Lancet Infect Dis**, v. 3, p. 159-166, 2008.

PIVELI, R. P.; KATO, M. T. **Qualidade das águas e poluição: Aspectos Físico-Químicos**, 1ª ed., cap. 6 e 7. ABES-Associação Brasileira de Engenharia Sanitária e Ambiental, São Paulo, 2006.

POIREL, L. et al. Multiplex PCR for detection of acquired carbapenemase genes. **Diagn Microbiol Infect Dis**, v. 70, p. 119-123, 2011.

POLOTTO, M. et al. Detection of *P. aeruginosa* harboring bla. **BMC Infectious Diseases**, v. 12, p. 176, 2012.

POPOVIC, M. et al. Fosfomycin: an old, new friend? **Eur J Clin Microbiol Infect Dis**, v. 29, p. 127-142, 2010.

PRADO, T. et al. Detection of extended-spectrum β-lactamase-producing *Klebsiella pneumoniae* in effluents and sludge of a hospital sewage treatment plant. **Lett Appl Microbiol**, v. 46, p. 136-141, 2008.

PRAYITNO, K. Z. et al. Hospital wastewater treatment using aerated fixed film biofilter-ozonation (Af2b/O3). **Adv Environ Biol**, v. 8, p. 1251–1259, 2014.

QUINTERA, S.; PEIXE, L. Multiniche screening reveals the clinically relevant metallo-b-lactamase VIM-2 in *Pseudomonas aeruginosa* far from the hospital setting: an ongoing dispersion process? **Appl Environ Microbiol**, v. 72, p. 3743-3745, 2006.

RIBEIRO FILHO, V. O. Gerenciamento de Resíduos de Serviços de Saúde. In: FERNANDES, A. T. **Infecção hospitalar e suas interfaces na área da saúde**. São Paulo, Atheneu, v. 2, p. 1156-1200, 2000.

RIBEIRO, M. C. S.; BERTOLOZZI, M. R. A questão ambiental como objeto de atuação da vigilância Sanitária: uma análise da inserção das enfermeiras nesse campo. **Rev Latino-am Enfermagem**, v. 12, p. 736-744, 2004.

ROBLEDO, I. E.; AQUINO, E. E.; VÁZQUEZ, G. J. Detection of the KPC gene in *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii* during a PCR-based nosocomial surveillance study in Puerto Rico. **Antimicrob Agents Chemother**, v. 55, p. 2968-2970, 2011.

ROMÃO, C. et al. Presence of qacEΔ1 gene and susceptibility to a hospital biocide in clinical isolates of *Pseudomonas aeruginosa* resistant to antibiotics. **Current Microbiology**, v. 63, p. 16 - 21, 2011.

SABAT, A. J. et al. Overview of molecular typing methods for outbreak detection and epidemiological surveillance. **Eurosurveillance**, v. 18, p. 17-30, 2013.

SADER, H. S. et al. IMPs, VIMs and SPMs: the diversity of metallo-beta-lactamases produced by carbapenem-resistant *Pseudomonas aeruginosa* in a Brazilian hospital. **Clin Microbiol Infect**, v. 11, p. 73-76, 2005.

SANTORO, D. O. **Avaliação da influência do tratamento do resíduo líquido hospitalar em isolados de *Pseudomonas aeruginosa* e na diversidade de *Pseudomonas* spp.** 2011. 112p. Dissertação (Mestrado em Vigilância Sanitária) – Instituto Nacional de Controle de Qualidade em Saúde, Fundação Oswaldo Cruz, Rio de Janeiro, 2011.

SANTORO, D. O.; ROMÃO, C. M.; CLEMENTINO, M. M. Decreased aztreonam susceptibility among *Pseudomonas aeruginosa* isolates from hospital effluent treatment system and clinical samples. **Int J Env Health Res**, v. 22, p. 560-570, 2012.

SHRESTHA, R. R.; HABERL, R.; LABER, J. Constructed wetland technology transfer to Nepal. **Water Sci Technol**, v. 43, p. 345–350, 2001.

SILBERT, S. et al. Evaluation of three molecular typing techniques for no fermentative Gram-negative bacilli. **Infection Control and Hospital Epidemiology**, v. 25, p. 847-851, 2004.

SILBY, M. W. et al. *Pseudomonas* genomes: diverse and adaptable. **FEMS Microbiol Rev**, v. 35, p. 652-680, 2011.

SILVA, M. T. N. **Prevalência de beta-lactamases de amplo espectro e metilases RNAr 16S em isolados clínicos de *Pseudomonas aeruginosa* multirresistentes recuperados em diferentes hospitais de São Paulo.** 2009. 83f. (Mestrado em Análises Clínicas) – Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, 2009.

SILVA, K. C.; LINCOPAN, N. Epidemiologia das beta-lactamases de espectro estendido no Brasil: impacto clínico e implicações para o agronegócio. **J Bras Patol Med Lab**, v. 48, p. 91-99, 2012.

SILVEIRA, I. C. T.; MONTEGGIA, L. O. O uso de contadores biológicos rotatórios no tratamento de efluente hospitalar. In: **22º Congresso Brasileiro de Engenharia Sanitária e Ambiental-ABES**, Joinville - SC, 2003.

SIPIBEL REPORT. **Effluents hospitaliers et stations d'épuration urbaines: caractérisation, risques et traitabilité- Presentation and premiers resultants**. Disponível em: http://www.graie.org/Sipible/publications.html (Acesso em: 20 out. 2015).

SYKES, R. B.; MATTEW, M. The β-lactamases of Gram-negative bacteria and their rôle in resistance to β-lactam antibiotics. **J Antimicrob Chemother**, v. 2, p. 115-157, 1976.

SPILKER, T. et al. PCR-based assay for differentiation of *Pseudomonas aeruginosa* from other *Pseudomonas* species recovered from cystic fibrosis patients. **J Clin Microbiol**, v. 42, p. 2074-2079, 2004.

STOKES, H. W.; HALL, R. M. A novel family of potentially mobile DNA elements encoding site-specific gene-integration functions: integrons. **Mol Microbiol**, v. 3, p. 1669-1683, 1989.

TAKAHATA, S. et al. Molecular mechanisms of fosfomycin resistance in clinical isolates of *Escherichia coli***. Int J Antimicrob Agents**, v. 35, p. 333-337, 2010.

TAMURA, K. et al. MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. **Molecular Biology and Evolution**, v. 28, p. 2731-2739, 2011.

TANNER, C. C.; HEADLEY, T. R. Components of floating emergent macrophyte treatment wetlands influencing removal of stormwater pollutants. **Ecological Engineering**, v. 37, p. 474-486, 2011.

TOLEMAN, M. A. et al. Molecular characterization of SPM-1, a novel metallo-β-lactamase isolated in Latin America: report from the SENTRY Antimicrobial Surveillance Program. **J Antimicrob Chemother**, v. 50, p. 673-679, 2002.

TOLLENTINO, F. M. et al. High Prevalence of *bla*$_{CTX-M}$ Extended Spectrum Beta-Lactamase Genes in *Klebsiella pneumoniae* Isolates from a Tertiary Care Hospital: First report of *bla*$_{SHV-12}$, *bla*$_{SHV-31}$, *bla*$_{SHV-38}$, and *bla*$_{CTX-M-15}$ in Brazil. **Microb Drug Resist**, v. 17, p. 7–16, 2011.

TSUKAYAMA, D. T. et al. The evolution of *Pseudomonas aeruginosa* during antibiotic rotation in a medical intensive care unit: the RADAR-trial. **Int J Antimicrob Agents**, v. 24, p. 339-345, 2004.

TZOUVELEKIS, L. S. et al. CTX – M - type - beta-lactamases: an emerging group of extended spectrum enzymes. **Int J Antimicrob Agents**, v. 14, p. 137-142, 2000.

VAN BELKUM, A. et al. Guidelines for the validation and application of typing methods for use in bacterial epidemiology. **Clin Microbiol Infect Dis**, v. 13, p. 1–46, 2007.

VARELA, A. R. et al. Insights into the relationship between antimicrobial residues and bacterial populations in a hospital-urban wastewater treatment plant system. **Water Res**, v. 54, p. 327–336, 2014.

VERLICCHI, P. et al. Hospital effluents as a source of emerging pollutants: An overview of micropollutants and sustainable treatment options. **Journal of Hydrology**, v. 389, p. 416- 428, 2010.

VERSALOVIC, J.; KOEUTH, T.; LUPSKI, J. R. Distribution of repetitive DNA sequences in eubacterial and application to fingerprinting of bacterial genomes. **Nucleic Acids Res**, v. 19, p. 6823-6831, 1991.

WALSH, T. R. Emerging carbapenemases: a global perspective. **Int J Antimicrob Agents**, v. 36, p. S8-S14, 2010.

WANG, X. R. et al. Prevalence and characterization of plasmid-mediated *bla*$_{ESBL}$ with their genetic environment in *Escherichia coli* and *Klebsiella pneumoniae* in patients with pneumonia. **Chin Med J**, v. 125, p. 894-900, 2012.

WELDHAGEN, G. F.; POIREL, L.; NORDMANN, P. Ambler class A extended-spectrum beta-lactamases in *Pseudomonas aeruginosa*: novel developments and clinical impact. **Antimicrobial Agents and Chemotherapy**, v. 47, p. 2385-2392, 2003.

WILKE, M. S.; LOVERING, A. L.; STRYNADKA, N. C. J. β-Lactam antibiotic resistance: a current structural perspective. **Current Opinion in Microbiology**, v. 8, p. 525-533, 2005.

WILSON, L. A.; SHARP, P. M. Enterobacterial repetitive intergenic consensus (ERIC) sequences in *Escherichia coli*: Evolution and implications for ERIC-PCR. **Molecular Biology and Evolution**, v. 23, p. 1156-1168, 2006.

WITTE, W. Ecological impact of antibiotic use in animals on different complex microflora: environment. **International Journal of Antimicrobial Agents**, v. 14, p. 321-325, 2000.

YANG, C. M. et al. Comparison of antimicrobial resistance patterns between clinical and sewage isolates in a regional hospital in Taiwan. **Letters in Applied Microbiology**, v. 48, p. 560-565, 2009.

YONEDA, K. et al. Measurement of *Pseudomonas aeruginosa* multidrug efflux pumps by quantitative real-time polymerase chain reaction. **FEMS Microbiol Lett**, v. 243, p. 125-131, 2005.

ZAVASCKI, A. P. et al. High prevalence of metallo-beta-lactamase-mediated resistance challenging antimicrobial therapy against *Pseudomonas aeruginosa* in a Brazilian teaching hospital. **Epidemiol Infect**, v. 135, p. 343-345, 2007a.

ZAVASCKI, A. P. et al. Polymyxin B for the treatment of multidroga-resistant pathogens: a critical review. **J Antimicrob. Chemother**, v. 60, p. 1206-1215, 2007b.

ZAVASCKI, A. P. et al. Multidrug-resistant *Pseudomonas aeruginosa* and *Acinetobacter baumannii*: resistance mechanisms and implications for therapy. **Expert Rev Anti Infect Ther**, v. 8, p. 71-93, 2010.

APÊNDICE A - ARTIGO PUBLICADO NA JOURNAL OF APPLIED MICROBIOLOGY (2015)

Journal of Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Genotypic characteristics of multidrug-resistant *Pseudomonas aeruginosa* from hospital wastewater treatment plant in Rio de Janeiro, Brazil

C.C. Miranda[1], I. de Filippis[1], L.H. Pinto[2], T. Coelho-Souza[1], K. Bianco[1], L.C. Cacci[3], R.C. Picão[3] and M.M. Clementino[1]

1 Instituto Nacional de Controle de Qualidade em Saúde, FIOCRUZ, Rio de Janeiro, Brazil
2 Departamento de Bioquímica, Instituto de Biologia Roberto Alcântara Gomes, Universidade do Estado do Rio de Janeiro, Rio de Janeiro, Brazil
3 Instituto de Microbiologia Paulo de Góes, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil

Keywords
clonal complexes, hospital sewage, multidrug-resistant, multilocus sequence typing, *Pseudomonas aeruginosa*.

Correspondence
Maysa M. Clementino, Fundação Oswaldo Cruz, Instituto Nacional de Controle de Qualidade em Saúde. Avenida Brasil, 4.365 CEP: 21040-900, Rio de Janeiro-RJ, Brazil.
E-mail: maysa.mandetta@incqs.fiocruz.br

2014/2387: received 20 November 2014, revised 16 January 2015 and accepted 24 February 2015

doi:10.1111/jam.12792

Abstract

Aims: To investigate *Pseudomonas aeruginosa* isolates from a hospital wastewater treatment plant (HWTP), focusing on enzyme-based mechanisms of β-lactams resistance and the genetic relatedness among isolates.

Methods and Results: Forty-one *Ps. aeruginosa* strains recovered from a HWTP were identified by amplification of 16S rRNA gene. β-lactamase production was screened by disc diffusion, CHROMagar extended-spectrum β-lactamase (ESBL) and β-lactamase strips. β-lactamase and ESBL producing isolates were investigated by PCR for the presence of ESBL, metallo-β-lactamase and *Klebsiella pneumoniae* carbapenemase encoding genes. Thirty-four isolates (83%) were resistant to at least one antibiotic belonging to three or more classes. Out of these 34 isolates, 28 (82%) were classified as multidrug-resistant (MDR) and 6 (18%) extensively drug-resistant (XDR). Genetic relatedness by Enterobacterial Repetitive Intergenic Consensus sequence-PCR and Multilocus sequence typing analysis showed 20 distinct profiles and 15 sequencing types respectively. Clonal Complex 244 (CC244) shows the pathogenic potential of this clone carrying MDR and XDR strains from clinical, environmental and hospital waste sources.

Conclusions: Our results suggest that treatment facilities for hospital wastewater can stimulate the increase of antimicrobial resistance bacteria and genes.

Significance and Impact of the Study: The great genetic diversity of *Ps. aeruginosa* recovered from HWTP constantly released into aquatic systems allow the spread of antimicrobial-resistant organisms and genes.

Introduction

Pseudomonas aeruginosa is an opportunistic pathogen with intrinsic resistance to many antimicrobials. Furthermore, under selective pressure, this micro-organism may easily develop powerful resistance either by mutation in chromosomally encoded genes or by horizontal transfer of resistance genes (Zhao and Hu 2010). Infections caused by this pathogen are often difficult to treat because of its multidrug-resistant (MDR) phenotype (De Francesco *et al.* 2013).

Production of β-lactamases, such as extended-spectrum β-lactamases (ESBLs) and carbapenemases, is an important mechanism of β-lactam antibiotics resistance in *Ps. aeruginosa* nosocomial isolates, which jeopardizes antimicrobial therapy in hospitalized patients (Picão *et al.* 2009). Carbapenemase production is of particular concern as it confers resistance to all β-lactams including extended-spectrum cephalosporins, monobactams and carbapenems (Zavascki *et al.* 2010). Carbapenemases that were identified in *Ps. aeruginosa* so far include: metallo-β-lactamases (MBLs) of the VIM (Verona imipenemase),

IMP (imipenemase), SPM (São Paulo metallo β-lactamase), GIM (German imipenemase) AIM (Australian imipenemase), DIM (Dutch imipenemase) and NDM types (New Delhi metallo-β-lactamase); KPC (*Klebsiella pneumoniae* carbapenemase), GES (Guiana extended spectrum) types (GES-2, -4, -5, -6 and -11) (Poirel *et al.* 2002; Gupta 2008; Jovcic *et al.* 2011) and OXA (Oxacillinase) type carbapenemases (Poirel *et al.* 2007, 2010). Among these resistance mechanisms, SPM-1, VIM-2, IMP-1, IMP-16, GES-5 and KPC-2 (Toleman *et al.* 2002; Mendes *et al.* 2004; Sader *et al.* 2005; Martins *et al.* 2007; Picão *et al.* 2009; Silva *et al.* 2011; Jácome *et al.* 2012) were found in *Ps. aeruginosa* from Brazil, most of which were clinical isolates.

Hospital effluents may represent a great risk to human public health due to the presence of pathogens and chemicals such as disinfectants, anaesthetics, heavy metals, antimicrobial agents and other drugs that are not metabolized by patients (Emmanuel *et al.* 2005). The disposal of this liquid waste in the treatment of hospital wastewater treatment plant (HWTP) can favour, the selective pressure, an increase in bacterial populations with phenotypes of multidrug resistance to antimicrobials (Prado *et al.* 2008).

Many carbapenem-resistant *Ps. aeruginosa* isolates and clinically important ESBLs-and carbapenemase-encoding genes have been found in hospital wastewater (Santoro *et al.* 2012), hospital and municipal sewage (Korzeniewska *et al.* 2013), urban sewerage systems (containing rainwater, hospital and urban wastewater) (Slekovec *et al.* 2012) wastewater contaminated rivers (Fontes *et al.* 2011; Amos *et al.* 2014). Of notice, NDM-1-producing *Ps. aeruginosa* was identified in tap water collected in India (Walsh *et al.* 2011).

In order to circumvent this problem, hospital effluents have been subjected to different treatments. However, several studies have showed that antibiotic resistance bacteria and genes persist in effluents of a variety of full-scale managements at levels above those typical of aquatic environments, even after disinfection (Auerbach *et al.* 2007).

The aim of this study was to investigate the antimicrobial susceptibility profiles and genetic relatedness of *Ps. aeruginosa* isolates obtained from a HWTP. We also explored the β-lactam-encoding genes and the clonal relationships among the isolates using Multilocus sequence typing (MLST) scheme.

Materials and methods

Study setting and sewage sampling

The effluent treatment system studied is located at a healthcare complex in the metropolitan area of Rio de Janeiro city, Brazil. The facility holds 322 beds and receives around 30 000 persons per month. The hospital contains a wastewater treatment plant that treats 220 cubic meters of sewage per day. It performs tertiary treatment in four stages, including pretreatment to remove gross solids; an aeration tank (continuous stirred tank reactor with sludge recycle), a clarifier tank and a post-treatment (disinfection of final effluent by chlorination). The treated effluent is discharged in rainwater network and then, into water bodies such as rivers and seawater.

Samples were collected in 2008 and in 2010 from five steps across the station (Grid-affluent, aeration tank, sludge, chlorination tank and treated effluent). Five hundred millilitres per site were collected approximately 20 cm below the surface in sterile glass bottles. Samples were kept refrigerated (4°C) until processed in the laboratory, within 8 h of collection. Temperature, pH, conductivity, dissolved oxygen, turbidity and salinity was assessed at the time of sample collection using Water Quality Checker U-10 (HORIBA, Kyoto, Japan) and dosage of chlorine was carried out using chlorine meter (Homis, São Paulo, Brazil).

Isolation and identification of *Pseudomonas aeruginosa*

A 100 ml-aliquot of each sample was filtered on membranes of 0·45 μm porosity. The membranes were transferred to selective asparagine broth and were incubated at 37°C for 24 h. The appearance of green fluorescence under ultraviolet light was considered positive for *Ps. aeruginosa* growth. Aliquots of 1 ml from these tubes were transferred to acetamide broth and were incubated at 37°C for 24 h. Tubes in which the development of pink colour was observed had an aliquot streaked on acetamide broth (Fuentefria *et al.* 2011). After incubation at 37°C for 24 h, six colonies per plate were selected for further studies, comprising a total of 60 isolates. Isolates were identified phenotypically using Vitek 2 GNI cards following the manufacturer's recommendations (bioMerieux, Marcy L'etoile, France) and results were certified by *Ps. aeruginosa*-specific PCR analysis (Spilker *et al.* 2004).

Antimicrobial susceptibility and MDR/XDR classification

The isolates were tested for antibiotic susceptibility by disc diffusion according to the Clinical Laboratory Standard Institute (CLSI 2014). *Escherichia coli* (ATCC 25922) and *Ps. aeruginosa* (ATCC 27853) were used as quality control strains. Seventeen antimicrobial agents were tested: amikacin (30 μg), aztreonam (30 μg), cefepime (30 μg), cefotaxime (30 μg), ceftazidime (30 μg), ceftriaxone (30 μg), ciprofloxacin (5 μg) gentamicin (10 μg), imipenem (10 μg), meropenem (10 μg), levofloxacin (5 μg),

piperacillin/tazobactam (100/10 μg), ticarcillin/clavulanic acid (75/10 μg), tobramycin (10 μg), polymyxin B (300 units), colistin (10 μg) and fosfomycin (200 μg).

The isolates were classified as MDR and extensively drug-resistant (XDR) according to Magiorakos *et al.* (2012). Production of β-lactamases and ESBLs was investigated by β-lactamase strip (Probac, São Paulo, Brazil) and CHROMagar—ESBL (CHROMagar, Paris, France) respectively. Statistical significance ($P < 0{\cdot}05$) between resistance profiles of each step were inferred by Fisher or chi-square test.

Detection of ESBL, MBL and KPC genes

The β-lactamase and ESBL positive isolates were screened by PCR for the presence of ESBL (bla_{PER}, bla_{VEB}, bla_{SHV}, $bla_{CTX\text{-}M\text{-}1}$, $bla_{CTX\text{-}M\text{-}2}$, $bla_{CTX\text{-}M\text{-}8}$, $bla_{CTX\text{-}M\text{-}9}$, $bla_{CTX\text{-}M\text{-}25}$, bla_{TEM} and bla_{GES}), MBL (bla_{IMP}, bla_{VIM}, bla_{SPM} and bla_{NDM}) and KPC (bla_{KPC}) encoding genes, as previously described (Toleman *et al.* 2002; Nagano *et al.* 2004; Dubois *et al.* 2005; Poirel *et al.* 2011; Doyle *et al.* 2012; Monteiro *et al.* 2012; Wang *et al.* 2012; Ahmed *et al.* 2013; Barguigua *et al.* 2013).

ERIC typing analysis

Genotypic analysis of the strains was investigated by amplification of the Enterobacterial Repetitive Intergenic Consensus sequence (ERIC-PCR). The extraction of genomic DNA was performed using the protocol for Gram-negative Dnaeasy®Blood&Tissuet (Qiagen®, Valencia, CA, USA) according to the manufacturer's instructions and the primer ERIC2 were used for the amplification as previously described (Versalovic *et al.* 1991). The amplicons were analysed by gel electrophoresis and stained with ethidium bromide (3 mg ml^{-1}). The gel was photographed and analysed using ImageQuant300 (GE, Oppsala, Sweden). Band patterns were analysed with BIONUMERICS ver. 6.6 (Applied Maths, Kortrijk, Belgium) using the Dice coefficient and unweighted pair group method with arithmetic mean pair group method with arithmetic average. Isolates with 100% level of similarity were considered clonally related.

Multilocus sequence typing

The ancestral relationship of the *Ps. aeruginosa* isolates were analysed by MLST with a representative strain of each ERIC type. Most primers used for amplification and sequencing of seven housekeeping genes (*acs*A, *aro*E, *gua*A, *mut*L, *nuo*D, *pps*A and *trp*E) were designed (L. Cacci, personal communication) with primer-blast assistance (http://www.ncbi.nlm.nih.gov/tools/primer-blast) with exception of *acs*A-F (Curran *et al.* 2004). The amplification of the housekeeping genes was performed as previously described and the nucleotide sequences were sequenced with fluorescent terminators (BigDye; Applied Biosystems, Foster City, CA) on an Applied Biosystems ABI Prism 3730 automated DNA sequences in duplicate for each primer (Otto *et al.* 2008).

Nucleotide sequences were submitted to the MLST database to determine the allelic numbers and Sequence Types (ST). Association of related ST to form clonal complexes (CCs) based on the number of identical alleles shared by ST is not a well-defined rule and can be subjective. The *Ps. aeruginosa* MLST database describes only two CCs: PA01 associated with ST-549 and PA14 associated with ST-253 and no information is disclosed of how these CCs were formed as there is only one ST for each CC. Maâtallah *et al.* (2013) considered that ST sharing five or more identical alleles are part of the same CC.

The MLST profiles were clustered with the BIONUMERICS 6.6 software (Applied Maths, Sint-Martens-Latem, Belgium) using a categorical coefficient and graphing was assessed using the minimum spanning tree tool, as described before (Schouls *et al.* 2004).

Results

Physical and chemical parameters

The pH of the five steps of the first sample analysed was maintained between 6·0–6·8, while in the second sample between 7·6 and 8·4, thus within the range of pH that enables the growth of many micro-organisms, including *Ps. aeruginosa*. Turbidity showed high values in step 1 and step 2 both samplings, mainly due to the presence of suspended solids in the water. The dissolved oxygen levels had the highest in step 2 where aeration occurs and elevates the levels of Dissolved Oxygen (DO), which is essential for organic matter biodegradation. The temperature of the five steps of two samplings remained at 28–30°C favouring mesophilic forms present. The chlorine concentration was high enough at the step 4 which the liquid chlorine is added to the tank. Mainly due to the presence of hypochlorite ion in high concentration, the conductivity was also high at this point that is directly proportional to the ionization of substances dissolved in the liquid (Table 1).

Pseudomonas aeruginosa identification and antimicrobial susceptibility profile

The five steps of sewage treatment at the HWTP yielded 60 isolates, 41 of which were identified as *Ps. aeruginosa*: step 1 – Grid/affluent ($n = 6$); step 2 – aeration tank ($n = 9$); step 3 – sludge ($n = 10$); step 4 – chlorination tank ($n = 4$); step 5 – treated effluent ($n = 12$).

Disc-diffusion susceptibility testing indicated high resistance to various antimicrobial agents. Highest resistance rate was observed for fosfomycin (88%) followed by ticarcillin/clavulanic acid (71%), ceftriaxone (63%), aztreonam (59%), cefotaxime (54%), imipenem (39%), cefepime (24%), meropenem (22%), ceftazidime (20%), tobramycin (20%), polymyxin B (20%), colistin (17%), gentamicin (15%), ciprofloxacin (12%), levofloxacin (12%), piperacillin/tazobactam (12%) and amikacin 2%). Susceptibility levels to fosfomycin, tobramycin, levofloxacin, ciprofloxacin and piperacillin/tazobactam were lower for the first three steps of effluent treatment (grid, aeration and sludge), with significant increase ($P < 0{\cdot}05$) on the fourth and fifth steps (chlorination and treated effluent).

Thirty-four out of 41 *Ps. aeruginosa* (83%) presented multidrug resistance profiles and were classified as MDR (82%) and XDR (18%) distributed along the five-step points of the HWTP. Thirty-eight isolates (93%) produced β-lactamase enzymes with 29 (76·3%) ESBL (Fig. 1).

ESBL, KPC and MBL-encoding genes

Among 29 (71%) isolates exhibiting ESBL phenotype, 13 harboured bla_{TEM} (45%), 7 harboured bla_{SHV} (24%) and 1 harboured $bla_{\text{CTX-M-1}}$ (3·5%). It is important to emphasize, however, that we did not sequence the amplicons obtained, thus temoniera (TEM) and sulfhydryl variable (SHV) production may not necessarily confer the ESBL phenotype observed. Thirty-eight (93%) isolates showed antimicrobial resistance for carbapenems, 14 carried bla_{KPC} (37%), 14 (37%) presented bla_{VIM} and 6 presented bla_{SPM} (18%) (Table 2).

ERIC and MLST genotyping

ERIC-PCR was able to fingerprint and assign a specific profile to all *Ps. aeruginosa* strains studied from different steps from a HWTP. Among all studied strains, ERIC-PCR resolved 20 genotypes. The banding patterns were highly reproducible under visual and automated analysis (Fig. 2).

MLST analysis revealed a total of 15 different ST, with five previously described (ST-238, ST-244, ST-381, ST-595 and ST-1621) and 10 new ST which were deposited in the *Ps. aeruginosa* MLST database (ST-1853, ST-1854, ST-1855, ST-1856, ST-1857, ST-1858, ST-1859, ST-1860, ST-1861, ST-1862) (Table 3). Discriminatory power of ERIC-PCR was higher than MLST as strains belonging to the same ST showed different ERIC profiles. Non-MDR (resistant less than three antibiotics) isolates belonged to single ST (238 and 381), with the exception of ST-244, ST-1621 and ST-1862. ST-244 was present in four of the five steps of HWTP, while the new ST-1859 was present in two steps. These two ST were represented by the larger number of isolates for each ($n = 8$) (Fig. 3). The 41 isolates were grouped into 15 different ST, with 10 newly described. Among the new ST, nine were associated with at least one MDR strain and one strain belonging to ST-1854 showed a XDR pattern. Other XDR strains were classified as ST-244 and ST-595 and were deposited in the *Ps. aeruginosa* MLST database.

Discussion

We investigated *Ps. aeruginosa* isolates from the HWPT and determined their antibiotic resistance profile, focusing in particular on enzyme-based mechanisms of resistance to β-lactams and in the determination of the genetic relatedness among isolates. Our results may suggest that wastewater treatment is followed by an increase in resistance profiles, although a quantitative study should be conducted to confirm this hypothesis. Nevertheless, it should be emphasized that Chagas *et al.* (2011) reported 48 and 21% of resistance to cefotaxime and ciprofloxacin, respectively, in Enterobacteriaceae isolates from hospital wastewater. Similar findings regarding *E. coli* from hospital wastewater being resistant to quinolones and cephalosporins have been

Table 1 Physical and chemical parameters of HWTP steps

	Abiotic parameters													
	pH		Conductivity $\mu S\ cm^{-1}$		Turbidity NTU		DO mg l^{-1}		Temperature °C		Salinity %		Chlorine ppm	
Site (step)	2008	2010	2008	2010	2008	2010	2008	2010	2008	2010	2008	2010	2008	2010
Grid (1)	6·8	7·6	0·39	0·82	18	10	4·4	4·5	29	28	0·0	0·0	1	0·0
Aeration (2)	6·1	7·9	0·31	0·38	26	99	7·6	9·2	30	29	0·0	0·0	1	−1
Sludge (3)	6·1	8·2	0·29	0·38	14	6	1·5	9·3	30	29	0·0	0·0	1	−1
Chlorination (4)	6·3	8·4	0·52	0·48	12	4	3·5	9·2	30	29	0·0	0·0	10	10·9
Treated Effluent (5)	6·0	8·4	0·29	0·35	13	7	3·4	3·7	30	29	0·0	0·0	1	0·01

$\mu S\ cm^{-1}$, micro-Siemens per centimetre; NTU, Nephelometric Turbidity Unit; mg l^{-1} Milligrams per litre; °C, Celsius degrees; %, Per cent; ppm, Parts per million; HWTP, hospital wastewater treatment plant.

Hospital wastewater treatment plant
Primary treatment
Secondary treatment
Tertiary treatment
Grid
Aeration
Sludge
Chlorination
Treated effluent
Rainwater Network
Step 1
Step 2
Step 3
Step 4
Step 5
STRAIN
FOSFO
TIM
CTR
AZT
CTX
IMI
CPM
MEM
CAZ
TOB
POL
COL
GEN
CIP
LEV
PTZ
AMI
Profile

Figure 1 Susceptibility of isolates of the five steps of hospital wastewater treatment plant. Filled square, antimicrobial resistance; empty square, antimicrobial susceptibility; β, Beta-lactamase producers; *, extended-spectrum β-lactamase producers; M, multidrug-resistance; X, extensively drug-resistant.

reported among others from Denmark (Jakobsen *et al.* 2008), China (Han *et al.* 2012) and Polland (Korzeniewska and Harnisz 2013). Thus, HWTPs could be a hot spot for antibiotic-resistant bacteria selection (Pruden *et al.* 2013).

Table 2 β-lactamase genes detected in all the isolates of *Pseudomonas aeruginosa*

Isolates	β-lactamase genes	Isolation step
P3229	bla_{SHV}, bla_{KPC}	1
P3233	–	2
P3234	bla_{TEM}	3
P3238	bla_{TEM}, bla_{SPM}, bla_{VIM}	4
P3239	bla_{TEM}, bla_{VIM}	4
P3240	bla_{TEM}, bla_{KPC}, bla_{VIM}	5
P3241	–	5
P3242	–	5
P3246	bla_{TEM}	5
P3249	bla_{SHV}, bla_{VIM}	2
P3252	bla_{SHV}, $bla_{KPC,}$ bla_{VIM}	4
P3671	–	1
P3672	bla_{SPM}	1
P3673	bla_{SPM}	1
P3674	$bla_{SHV,}$ bla_{SPM}	1
P3675	bla_{VIM}	1
P3676	bla_{TEM}	2
P3677	bla_{KPC}, bla_{VIM}	2
P3678	bla_{SHV}, $bla_{CTX\text{-}M\text{-}1}$,$bla_{KPC}$	2
P3679	bla_{KPC}	2
P3680	bla_{KPC}	2
P3681	bla_{KPC}	2
P3682	bla_{KPC}	3
P3683	–	3
P3684	–	3
P3685	–	3
P3686	bla_{TEM}	3
P3687	bla_{KPC}	3
P3688	bla_{KPC}	3
P3689	bla_{TEM}, bla_{KPC}	3
P3691	bla_{TEM}	5
P3692	bla_{TEM}, bla_{VIM}	5
P3693	bla_{VIM}	5
P3694	$bla_{VIM,}$ bla_{SPM}	5
P3695	$bla_{VIM,}$ bla_{SPM}	5
P3696	–	5
P3697	–	5
P3846	bla_{TEM}, bla_{SHV}, $bla_{KPC,}$ bla_{VIM}	2
P3847	$bla_{KPC,}$ bla_{VIM}	3
P3848	bla_{TEM}, bla_{VIM}	4
P3849	bla_{TEM}	5

Isolation steps: (1) Grid/affluent; (2) aeration tank; (3) Sludge; (4) Chlorination tank; (5) Treated effluent.

Pseudomonas aeruginosa strains were recovered from all five stages of the HWTP studied, indicating that this species could be considered a good marker for studies aimed at evaluating the impact of wastewater treatment process on the prevalence of antibiotic resistance organisms and genes in the effluent. All stages of the station showed isolates exhibiting MDR pattern, in contrast, Slekovec *et al.* (2012), did not find resistant neither MDR isolates in HWTP before its release to the environment and in the sludge produced by the waste plant in France.

It is noteworthy to emphasize that 100% of the isolates of the chlorination step showed XDR profile. Using chemical disinfection to inactivate pathogens also plays an important role in controlling antibiotic-resistant bacteria in HWTPs. Studies on the effect of chlorination on antibiotic-resistant bacteria can be traced back to 1970s, where chlorination was shown to influence the proportion of multiple-antibiotic-resistant bacteria in drinking water and wastewater (Grabow *et al.* 1973; Armstrong *et al.* 1982; Murray *et al.* 1984). On the other hand, in a previous study, we demonstrated the absence of microbial growth in the chlorination step. However, *Ps. aeruginosa* cells detected in the following stage of the treatment revealed the capacity of regrowth in chlorinated sewage effluent maybe due to the bacteriostatic effect of chlorine, which justifies the presence of viable cells in stage 5 (Santoro *et al.* 2012).

Among strains exhibiting β-lactamase encoding genes, bla_{TEM}, bla_{SHV} $bla_{CTX\text{-}M\text{-}1}$, $bla_{KPC,}$ bla_{VIM} and bla_{SPM} were characterized randomly in 78% of the isolates. It is necessary to highlight that isolates carring bla_{TEM}, $bla_{KPC,}$ bla_{VIM} and bla_{SPM} were found in the fifth and final stage of the hospital wastewater with potential to spread throughout the aquatic environment, thus enabling human exposure and transmission.

Quintera *et al.* (2005) reported the isolation of a *Pseudomonas pseudoalcaligenes* VIM-2 strain from hospital sewage. This discovery suggests that the ongoing spread of the $bla_{VIM\text{-}2}$ is occurring simultaneously in several dimensions, as it can now be found in different environments and in several bacterial species. Another study revealed the presence of $bla_{VIM\text{-}2}$ in two unrelated *Ps. aeruginosa* isolates from aquatic environments (Quintera and Peixe 2006). The $bla_{SPM\text{-}1MBL}$ gene is the most prevalent in Brazil; its presence was evaluated in *Ps. aeruginosa* isolates from hospital sewage and surface-water samples, in order to obtain epidemiological data on the dissemination of this gene in environmental samples in southern Brazil (Fuentefria *et al.* 2009). The presence of

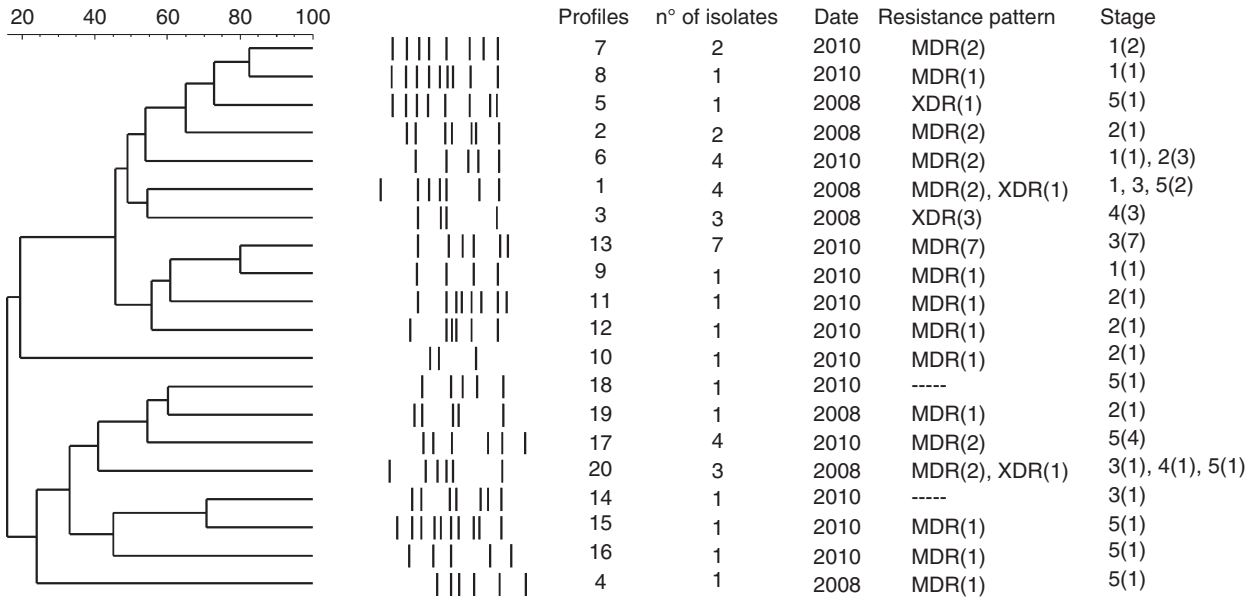


Figure 2 Cluster analysis by enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR) fingerprint (ERIC 2) of 41 *Pseudomonas aeruginosa* isolates. Clustering analysis was performed with aid of BIONUMERICS 6.6 (Applied Maths) and based on the Dice similarity coefficient and the unweighted pair group method with arithmetic mean.

Table 3 MLST of isolates representing 20 *Pseudomonas aeruginosa* distinct profiles from hospital wastewater treatment plants during 18-month period

				Allelic profile						
ERIC profile	No of isolates	Collected step	ST	*acs*A	*aro*E	*gua*A	*mut*L	*nuo*D	*pps*A	*trp*E
1	4	1, 3, 5	244	17	5	12	3	14	4	7
2	2	2	1853	16	22	11	5	4	4	10
3	3	4	244	17	5	12	3	14	4	7
4	1	5	244	17	5	12	3	14	4	7
5	1	5	1854	17	5	44	110	14	81	7
6	4	1, 2	1621	5	54	99	48	1	6	3
7	2	1	1855	5	54	44	48	14	81	3
8	1	1	1856	30	2	44	110	1	97	7
9	1	1	1857	28	54	44	110	4	79	3
10	1	2	1858	17	2	11	3	81	38	3
11	1	2	1859	17	5	36	7	27	4	5
12	1	2	1858	17	2	11	3	81	38	3
13	7	3	1859	17	5	36	7	27	4	5
14	1	3	381	11	20	1	65	4	4	10
15	1	5	1860	17	5	44	3	14	77	5
16	1	5	1861	17	20	49	110	11	79	10
17	4	5	1862	17	20	12	110	4	81	10
18	1	5	238	5	1	59	6	1	33	42
19	1	2	1853	16	22	11	5	4	4	10
20	3	3, 4, 5	595	17	5	12	5	14	4	7

ERIC, enterobacterial repetitive intergenic consensus; MLST, multilocus sequence typing; ST, sequence types.

pathogenic Enterobacteriaceae from a hospital effluent showed resistance to third-generation cephalosporins, cefepime, aminoglycosides, quinolones and a significant rate of carbapenem. Genes encoding ESBL (bla_{SHV}, bla_{TEM}, bla_{CTX-M} and bla_{GES}) were detected among 46 strains with reduced susceptibility to third-generation cephalosporins isolated from hospital sewage (Pitout *et al.* 2004; Coque *et al.* 2008; Naas *et al.* 2008).

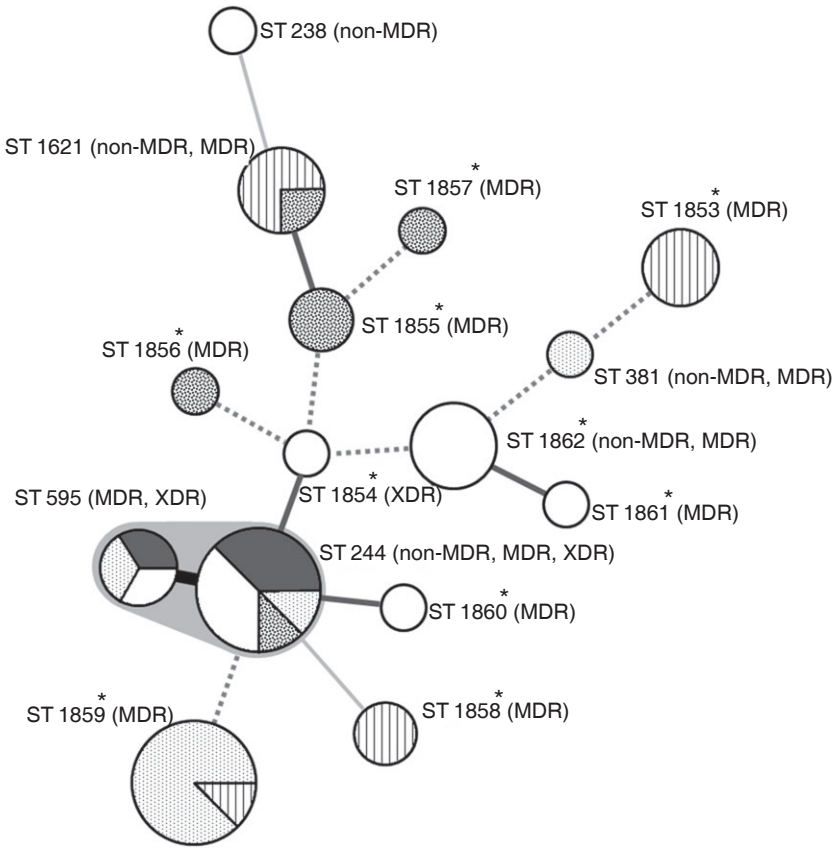


Figure 3 Minimum spanning tree of MLST sequence types of *Ps. aeruginosa* isolates from hospital wastewater treatment plant (HWTP). The tree was based on the analysis of concatenated sequences of acsA, aroE, guaA, mutL, nuoD, ppsA and trpE genes. Multidrug-resistant and extended drug-resistant strains are indicated beside each sequence type (ST). Circles indicate specific ST and the number of strains according to the size of the circle. Circle patterns indicate the HWTP step. New ST are marked with (*). Lines connecting ST groups, indicate that they differ in one allele (thick solid line), or three to five locus (thin and dotted lines). The halo surrounding ST-244 and ST-595 indicates a clonal complex (CC244). Relations between ST were determined by BioNumerics analysis (BIONUMERICS 6.6) where CC were formed by ST with single or double locus variation. (▩) Grid; (▥) Aeration; (▦) Sludge; (■) Chlorination and (□) Treated effluent.

The release of high concentrations of antibiotics and resistance genes in natural ecosystems is a recent event in evolutionary terms. However, these pollution can impact the structure and the activity of environmental microbial populations. Given that environmental micro-organisms are the original source of resistance genes acquired by human pathogens (Davies 1997). In water bodies, bacteria from different origins (human, animal, environmental) are able to mix, and resistance evolves as a consequence of promiscuous exchange and shuffling of genes and genetic vectors (Baquero *et al.* 2008).

The discharge of MDR bacteria including ESBL, KPC and MBL producers into an urban river is worrisome, as these isolates could persist in the environment and act as opportunistic pathogens and/or resistance reservoirs that could accelerate the evolution of antimicrobial resistance in the community (Kim and Aga 2007; Baquero *et al.* 2008; Martinez 2009).

The MLST analysis showed a high diversity, as reported in other previous studies (Gomila *et al.* 2013). ST244 is well distributed worldwide with 34 isolates in the MLST database being four isolated in Brazil (three from this study) and one clinical sample (Maâtallah *et al.* 2013; Bae *et al.* 2014; Chen *et al.* 2014;). Two strains belonging to ST595 deposited in the MLST database were isolated in Brazil both from nonclinical samples (one from this study). It is noteworthy to point out that the chlorination step showed the greatest number of isolates of MDR and XDR strains belonging to CC244 where all the isolates from this step were grouped (Fig. 3). To our knowledge, this is the first MLST analysis of *Ps. aeruginosa* isolated from hospital effluent.

Following the rule of Maâtallah *et al.* (2013), 11 CC, sharing five or more identical alleles, were described with two major clones formed by five ST each. One of these CC (CC244) named by its central ST-244, included other closely related ST (ST-990, ST-993, ST-986 and ST-654). Two ST belonging to CC244 (ST-244 and ST-654), included several clinical strains showing reduced susceptibility against different classes of antibiotics such as ESBL, IMP-type metallo-beta-lactamase and KPC producing. We could not compare strains associated with ST-990, ST-986 and ST-654 also included in CC244, as these ST were deleted from the MLST database and no data associated with these strains was available.

The three strains classified as ST-595 were beta-lactamase producers and showed reduced susceptibility with one strain showing a KPC and metallo-beta-lactamase phenotype (MDR), one ESBL and metallo-beta-lactamase (XDR) and one ESBL producer (MDR). Other eight

strains classified as ST-244 and also included in CC244 showed similar susceptibility patterns with several MDR and XDR strains KPC, ESBL and metallo-beta-lactamase producers. To date, CC244 has been detected among strains isolated from different sources such as clinical and environmental (Cholley *et al.* 2014). We first report, to our knowledge, the presence of strains belonging to CC244 from hospital effluent. These data suggest a possible turnover of strains sharing different antibiotic resistance mechanisms regardless of environment location.

The presence of MDR and XDR strains into CC244 suggests the pathogenic potential of this clone carrying strains with reduced susceptibility to several antimicrobial classes spreading through different sources from clinical to hospital effluent and the environment.

The spreading of MDR organisms and genes through the environment is worrying. Strategies to reduce this picture are required, including neutralization of antibiotics in wastewater and in the environment (Zwiener and Frimmel 2000; Ternes *et al.* 2003; Berglund *et al.* 2014).

Our results demonstrated the presence of isolates carrying important resistance mechanisms with potential to contaminate aquatic environments with increasing antibiotic resistance within the microbial community, leading to negative impacts on the environment and human health.

Acknowledgements

The authors are grateful to the Plataforma Genomica-Sequenciamento de DNA-RPT01A-PDTIS/FIOCRUZ. We acknowledge the financial support from CNPq (474204/2012-0), FAPERJ (E-26/112.657/2012) and INCQS-FIOCRUZ.

Conflict of Interest

No conflict of interest declared.

References

Ahmed, O.I., EL-Hady, S.A., Ahmed, T.M. and Ahmed, I.Z. (2013) Detection of bla_{SHV} and bla_{CTX-M} genes in ESBL producing *Klebsiella pneumoniae* isolated from Egyptian patients with suspected nosocomial infections. *Egypt J Med Hum Genet* **14**, 277–283.

Amos, G.C., Hawkey, P.M., Gazem, W.H. and Wellington, E.M. (2014) Waste water effluent contributes to the dissemination of CTX-M-15 in the natural environment. *J Antimicrob Chemother* **69**, 1785–1791.

Armstrong, J.L., Calomiris, J.J. and Seidler, R.J. (1982) Selection of antibiotic-resistant standard plate count bacteria during water treatment. *Appl Environ Microbiol* **44**, 308–316.

Auerbach, E.A., Seyfried, E.E. and McMahon, K.D. (2007) Tetracycline resistance genes in activated sludge wastewater treatment plants. *Water Res* **41**, 1143–1151.

Bae, I.K., Suh, B., Jeong, S.H., Wang, K.K., Kim, Y.R., Yong, D. and Lee, K. (2014) Molecular epidemiology of *Pseudomonas aeruginosa* clinical isolates from Korea producing β-lactamases with extended-spectrum activity. *Diagn Microbiol Infect Dis* **79**, 373–377.

Baquero, F., Martinez, J.L. and Canton, R. (2008) Antibiotics and antibiotic resistance in water environments. *Curr Opin Biotechnol* **19**, 260–265.

Barguigua, A., El Otmani, F., Talmi, M., Reguig, A., Jamali, L., Zerouali, K. and Timinouni, M. (2013) Prevalence and genotypic analysis of plasmid-mediated β-lactamases amongurinary *Klebsiella pneumoniae* isolates in Moroccan community. *J Antibiot* **66**, 11–16.

Berglund, B., Khan, G.A., Weisner, S.E., Ehde, P.M., Fick, J. and Lindgren, P.E. (2014) Efficient removal of antibiotics in surface-flow constructed wetlands, with no observed impact on antibiotic resistance genes. *Sci Total Environ* **1**, 476–477.

Chagas, T.P., Seki, L.M., Cury, J.C., Oliveira, J.A., Dávila, A.M., Silva, D.M. and Asensi, M.D. (2011) Multiresistance, beta-lactamase-encoding genes and bacterial diversity in hospital wastewater in Rio de Janeiro, Brazil. *J Appl Microbiol* **111**, 572–581.

Chen, Y., Sun, M., Wang, M., Lu, Y. and Yan, Z. (2014) Dissemination of IMP-6-producing *Pseudomonas aeruginosa* ST244 in multiple cities in China. *Eur J Clin Microbiol Infect Dis* **33**, 1181–1187.

Cholley, P., Ka, R., Guyeux, C., Thouverez, M., Guessennd, N., Ghebremedhin, B., Frank, T., Bertrand, X. *et al.* (2014) Population structure of clinical *Pseudomonas aeruginosa* from West and Central African countries. *PLoS ONE* **9**(9), e107008.

Clinical and Laboratory Standards Institute (2014) *Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Third Informational Supplement*. CLSI document M100–S23. Wayne, PA: CLSI.

Coque, T.M., Baquero, F. and Canton, R. (2008) Increasing prevalence of ESBL-producing Enterobacteriaceae in Europe. *Euro Surveill* **13**, n.47.

Curran, B., Jonas, D., Grundmann, H., Pitt, T. and Dowson, C.G. (2004) Development of a multilocus sequence typing scheme for the opportunistic pathogen *Pseudomonas aeruginosa*. *J Clin Microbiol* **42**, 5644–5649.

Davies, J.E. (1997) Origins, acquisition and dissemination of antibiotic resistance determinants. *Ciba Found. Symp.* **207**, 15–27.

De Francesco, M.A., Ravizzola, G., Peroni, L., Bonfanti, C. and Manca, N. (2013) Prevalence of multidrug-resistant *Acinetobacter baumannii* and *Pseudomonas aeruginosa* in an Italian hospital. *J Infect Public Health* **6**, 179–185.

Doyle, D., Peirano, G., Lascols, C., Lloyd, T., Church, D.L. and Pitout, J.D. (2012) Laboratory detection of Enterobacteriaceae that produce carbapenemases. *J Clin Microbiol* **50**, 3877–3880.

Dubois, V., Arpin, C., Noury, P., Andre, C., Coulange, L. and Quentin, C. (2005) Prolonged outbreak of infection due to TEM-21-producing strains of *Pseudomonas aeruginosa* and enterobacteria in a nursing home. *J Clin Microbiol* **43**, 4129–4138.

Emmanuel, E., Perrodin, Y., Keck, G., Blanchard, J.M. and Vermande, P. (2005) Ecotoxicological risk assessment of hospital wastewater: a proposed framework for raw effluents discharging into urban sewer network. *J Hazardous Mat* **117**, 1–11.

Fontes, L.C., Neves, P.R., Oliveira, S., Silva, K.C., Hachich, E.M., Sato, M.I. and Lincopan, N. (2011) Isolation of *Pseudomonas aeruginosa* coproducing metallo-β-lactamase SPM-1 and 16S rRNA methylase RmtD1 in an urban river. *Antimicrob Agents Chemother* **55**, 3063–3064.

Fuentefria, D.B., Ferreira, A.E., Gräf, T. and Corção, G. (2009) Spread of Metallo-β-lactamases: screening reveals the presence of a *blaSPM-1* gene in hospital sewage in southern Brazil. *Braz J Microbiol* **40**, 82–85.

Fuentefria, A.D.B., Ferreira, A.E. and Corção, G. (2011) Antibiotic-resistant *Pseudomonas aeruginosa* from hospital wastewater and superficial water: are they genetically related? *J Environ Manage* **92**, 250–255.

Gomila, M., Del Carmen Gallegos, M., Fernández-Baca, V., Pareja, A., Pascual, M., íaz-Antolín, P., García-Valdés, E. and Lalucat, J. (2013) Genetic diversity of clinical *Pseudomonas aeruginosa* isolates in a public hospital in Spain. *BMC Microbiol* **13**, 138.

Grabow, W.O.K., Middendorff, I.G. and Prozeskv, O.W. (1973) Survival in maturation ponds of coliform bacteria with transferable drug resistance. *Water Res* **7**, 1589–1597.

Gupta, V. (2008) Metallo beta lactamases in *Pseudomonas aeruginosa* and *Acinetobacter* species. *Expert Opin Investig Drugs* **17**, 131–143.

Han, N., Sheng, D. and Xu, H. (2012) Role of *Escherichia coli* strain subgroups, integrons, and integron-associated gene cassettes in dissemination of antimicrobial resistance in aquatic environments of Jinan, China. *Water Sci Technol* **66**, 2385–2392.

Jácome, P.R., Alves, L.R., Cabral, A.B., Lopes, A.C. and Maciel, M.A. (2012) First report of KPC-producing *Pseudomonas aeruginosa* in Brazil. *Antimicrob Agents Chemother* **56**, 4990.

Jakobsen, L., Sandvang, D., Hansen, L.H., Bagger-Skjot, L., Westh, H., Jorgensen, C., Hansen, D.S., Pedersen, B.M. *et al.* (2008) Characterisation, dissemination and persistence of gentamicin resistant *Escherichia coli* from a Danish university hospital to the wastewater environment. *Environ Int* **34**, 108–115.

Jovcic, B., Lepsanovic, Z., Suljagic, V., Rackov, G., Begovic, J., Topisirovic, L. and Kojic, M. (2011) Emergence of NDM-1 metallo-β-lactamase in *Pseudomonas aeruginosa* clinical isolates from Serbia. *Antimicrob Agents Chemother* **55**, 3929–3931.

Kim, S. and Aga, D.S. (2007) Potential ecological and human health impacts of antibiotics and antibiotic-resistant bacteria from wastewater treatment plants. *J Toxicol Environ Health B Crit Rev* **10**, 559–573.

Korzeniewska, E. and Harnisz, M. (2013) Beta-lactamase producing Enterobacteriaceae in hospital effluents. *J Environ Manage* **123**, 1–7.

Korzeniewska, E., Korzeniewska, A. and Harnisz, M. (2013) Antibiotic resistant *Escherichia coli* in hospital and municipal sewage and their emission to the environment. *Ecotoxicol Environ Saf* **91**, 96–102.

Maâtallah, M., Bakhrouf, A., Habeeb, M.A., Turlej-Rogacka, A., Iversen, A., Pourcel, C., Sioud, O. and Giske, C.G. (2013) Four genotyping schemes for phylogenetic analysis of *Pseudomonas aeruginosa*: comparison of their congruence with multi-locus sequence typing. *PLoS ONE* **8**, e82069.

Magiorakos, A.P., Srinivasan, A., Carey, R.B., Carmeli, Y., Falagas, M.E., Giske, C.G., Harbarth, S., Hindler, J.F. *et al.* (2012) Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clin Microbiol Infect* **18**, 268–281.

Martinez, J.L. (2009) Environmental pollution by antibiotics and by antibiotic resistance determinants. *Environ Pollut* **157**, 2893–2902.

Martins, A.F., Zavascki, A.P., Gaspareto, P.B. and Barth, A.L. (2007) Dissemination of *Pseudomonas aeruginosa* producing SPM-1-like and IMP-1-like metallo-β-lactamases in hospitals from southern Brazil. *Infection* **35**, 457–460.

Mendes, R.E., Toleman, M.A., Ribeiro, J., Sader, H.S., Jones, R.N. and Walsh, T.R. (2004) Integron carrying a novel metallo-β-lactamase gene, blaIMP-16, and a fused form of aminoglycoside-resistant gene aac(6')-30/aac(6')-Ib': report from the SENTRY Antimicrobial Surveillance Program. *Antimicrob Agents Chemother* **48**, 4693–4702.

Monteiro, J., Widen, R.H., Pignatari, A.C., Kubasek, C. and Silbert, S. (2012) Rapid detection of carbapenemase genes by multiplex real-time PCR. *J Antimicrob Chemother* **67**, 906–909.

Murray, G.E., Tobin, R.S., Junkins, B. and Kushner, D.J. (1984) Effect of chlorination on antibiotic resistance pro files of sewage-related bacteria. *Appl Environ Microbiol* **48**, 73–77.

Naas, T., Poirel, L. and Nordmann, P. (2008) Minor extended-spectrum beta-lactamases. *Clin Microbiol Infect* **14**, 42–52.

Nagano, N., Nagano, Y., Cordevant, C., Shibata, N. and Arakawa, Y. (2004) Nosocomial transmission of CTX-M-2 beta-lactamase-producing *Acinetobacter baumannii* in a neurosurgery ward. *J Clin Microbiol* **42**, 3978–3984.

Otto, T.D., Vasconcellos, E.A., Gomes, L.H.F., Moreira, A.S., Degrave, W.M., Mendonça-Lima, L. and Alves-Ferreira,

M. (2008) ChromaPipe: a pipeline for analysis, quality control and management for a DNA sequencing facility. *Genet Mol Res* **7**, 861–871.

Picão, R.C., Poirel, L., Gales, L.C. and Nordmann, P. (2009) Diversity of β-lactamases produced by ceftazidime-resistant *Pseudomonas aeruginosa* isolates causing bloodstream infections in Brazil. *Antimicrob Agents Chemother* **53**, 3908–3913.

Pitout, J.D., Hossain, A. and Hanson, N.D. (2004) Phenotypic and molecular detection of CTX-M-beta-lactamases produced by *Escherichia coli* and *Klebsiella* spp. *J Clin Microbiol* **42**, 5715–5721.

Poirel, L., Weldhagen, G.F., De Champs, C. and Nordmann, P. (2002) A nosocomial outbreak of *Pseudomonas aeruginosa* isolates expressing the extended-spectrum beta-lactamase GES-2 in South Africa. *J Antimicrob Chemother* **9**, 561–565.

Poirel, L., Pitout, J.D. and Nordmann, P. (2007) Carbapenemases: molecular diversity and clinical consequences. *Future Microbiol* **2**, 501–512.

Poirel, L., Naas, T. and Nordmann, P. (2010) Diversity, epidemiology, and genetics of class D beta-lactamases. *Antimicrob Agents Chemother* **54**, 24–38.

Poirel, L., Revathi, G., Bernabeu, S. and Nordmann, P. (2011) Detection of NDM-1-producing *Klebsiella pneumoniae* in Kenya. *Antimicrob Agents Chemother* **55**, 934–936.

Prado, T., Pereira, W.C., Silva, D.M., Seki, L.M., Carvalho, A.P. and Asensi, M.D. (2008) Detection of extended-spectrum β-lactamase-producing *Klebsiella pneumoniae* in effluents and sludge of a hospital sewage treatment plant. *Lett Appl Microbiol* **46**, 136–141.

Pruden, A., Larsson, D.G., Amézquita, A., Collignon, P., Brandt, K.K., Graham, D.W., Lazorchak, J.M., Suzuki, S. *et al.* (2013) Management options for reducing the release of antibiotics and antibiotic resistance genes to the environment. *Environ Health Perspect* **121**, 878–885.

Quintera, S. and Peixe, L. (2006) Multiniche screening reveals the clinically relevant metallo-β-lactamase VIM-2 in *Pseudomonas aeruginosa* far from the hospital setting: an ongoing dispersion process? *Appl Environ Microbiol* **72**, 3743–3745.

Quintera, S., Ferreira, H. and Peixe, L. (2005) First isolation of *bla*VIM-2 in an environmental isolate of *Pseudomonas pseudoalcaligenes*. *Antimicrobial Agents Chemother* **49**, 2140–2141.

Sader, H.S., Reis, A.O., Silbert, S. and Gales, A.C. (2005) IMPs, VIMs and SPMs: the diversity of metallo-β-lactamases produced by carbapenem-resistant *Pseudomonas aeruginosa* in a Brazilian hospital. *Clin Microbiol Infect* **11**, 73–76.

Santoro, D.O., Romão, C.M. and Clementino, M.M. (2012) Decreased aztreonam susceptibility among *Pseudomonas aeruginosa* isolates from hospital effluent treatment system and clinical samples. *Int J Environ Health Res* **22**, 560–570.

Schouls, L.M., van der Heide, H.G., Vauterin, L., Vauterin, P. and Mooi, F.R. (2004) Multiple-locus variable-number tandem repeat analysis of Dutch *Bordetella pertussis* strains reveals rapid genetic changes with clonal expansion during the late 1990s. *J Bacteriol* **186**, 5496–5505.

Silva, F.M., Carmo, M.S., Silbert, S. and Gales, A.C. (2011) SPM-1-producing *Pseudomonas aeruginosa*: analysis of the ancestor relationship using multilocus sequence typing, pulsed-field gel electrophoresis, and automated ribotyping. *Microb Drug Resist* **17**, 215–220.

Slekovec, C., Plantin, J., Cholley, P., Thouverez, M., Talon, D., Bertrand, X. and Hocquet, D. (2012) Tracking down antibiotic-resistant *Pseudomonas aeruginosa* isolates in a wastewater network. *PLoS ONE* **7**, e49300.

Spilker, T., Coenye, T., Vandamme, E.P. and Lipuma, J.J. (2004) PCR-based assay for differentiation of *Pseudomonas aeruginosa* from other *Pseudomonas species* recovered from cystic fibrosis patients. *J Clin Microbiol* **42**, 2074–2079.

Ternes, T.A., Stüber, J., Herrmann, N., McDowell, D., Ried, A., Kampmann, M. and Teiser, B. (2003) Ozonation: a tool for removal of pharmaceuticals, contrast media and musk fragrances from wastewater? *Water Res* **37**, 1976–1982.

Toleman, M.A., Simm, A.M., Murphy, T.A., Gales, A.C., Biedenbach, D.J., Jones, R.N. and Walsh, T.R. (2002) Molecular characterization of SPM-1, a novel metallo-β-lactamase isolated in Latin America: report from the SENTRY Antimicrobial Surveillance Program. *J Antimicrob Chemother* **50**, 673–679.

Versalovic, J., Koeuth, T. and Lupski, J.R. (1991) Distribution of repetitive DNA sequences in eubacterial and application to fingerprinting of bacterial genomes. *Nucleic Acids Res* **19**, 6823–6831.

Walsh, T.R., Weeks, J., Livermore, D.M. and Toleman, M.A. (2011) Dissemination of NDM-1 positive bacteria in the New Delhi environment and its implications for human health: an environmental point prevalence study. *Lancet Infect Dis* **11**, 355–362.

Wang, X.R., Chen, J.C., Kang, Y., Jiang, N., An, S.C. and Gao, Z.C. (2012) Prevalence and characterization of plasmid-mediated blaESBL with their genetic environment in *Escherichia coli* and *Klebsiella pneumoniae* in patients with pneumonia. *Chin Med J* **125**, 894–900.

Zavascki, A.P., Carvalhaes, C.G., Picão, R.C. and Gales, A.C. (2010) Multidrug-resistant *Pseudomonas aeruginosa* and *Acinetobacter baumannii*: resistance mechanisms and implications for therapy. *Expert Rev Anti Infect Ther* **8**, 71–93.

Zhao, W.H. and Hu, Z.Q. (2010) Beta-lactamases identified in clinical isolates of *Pseudomonas aeruginosa*. *Crit Rev Microbiol* **36**, 245–258.

Zwiener, C. and Frimmel, F.H. (2000) Oxidative treatment of pharmaceuticals in water. *Water Res* **34**, 1881–1885.

APÊNDICE B - ARTIGO PUBLICADO NA FRONTIERS IN MICROBIOLOGY (2015)

frontiers in Microbiology

ORIGINAL RESEARCH
published: 20 August 2015
doi: 10.3389/fmicb.2015.00860

Isolation of aerobic cultivable cellulolytic bacteria from different regions of the gastrointestinal tract of giant land snail *Achatina fulica*

Guilherme L. Pinheiro[1,2], *Raquel F. Correa*[1], *Raquel S. Cunha*[1], *Alexander M. Cardoso*[1,3], *Catia Chaia*[4], *Maysa M. Clementino*[4], *Eloi S. Garcia*[1,5], *Wanderley de Souza*[1,2] *and Susana Frasés*[1,2]*

[1] *Diretoria de Metrologia Aplicada às Ciências da Vida, Instituto Nacional de Metrologia, Qualidade e Tecnologia, Rio de Janeiro, Brazil,* [2] *Laboratório de Ultraestrutura Celular Hertha Meyer, Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil,* [3] *Centro Universitário Estadual da Zona Oeste, Unidade Universitária de Biologia, Rio de Janeiro, Brazil,* [4] *Departamento de Microbiologia, Instituto Nacional de Controle da Qualidade em Saúde, Fundação Oswaldo Cruz, Rio de Janeiro, Brazil,* [5] *Departamento de Bioquímica e Biologia Molecular, Instituto Oswaldo Cruz, Fundação Oswaldo Cruz, Rio de Janeiro, Brazil*

The enzymatic hydrolysis of cellulose by cellulases is one of the major limiting steps in the conversion of lignocellulosic biomass to yield bioethanol. To overcome this hindrance, significant efforts are underway to identify novel cellulases. The snail *Achatina fulica* is a gastropod with high cellulolytic activity, mainly due to the abundance of glycoside hydrolases produced by both the animal and its resident microbiota. In this study, we partially assessed the cellulolytic aerobic bacterial diversity inside the gastrointestinal tract of *A. fulica* by culture-dependent methods and evaluated the hydrolytic repertoire of the isolates. Forty bacterial isolates were recovered from distinct segments of the snail gut and identified to the genus level by 16S rRNA gene sequence analysis. Additional phenotypic characterization was performed using biochemical tests provided by the Vitek2 identification system. The overall enzymatic repertoire of the isolated strains was investigated by enzymatic plate assays, containing the following substrates: powdered sugarcane bagasse, carboxymethylcellulose (CMC), p-nitrophenyl-β-D-glucopyranoside (pNPG), p-nitrophenyl-β-D-cellobioside (pNPC), 4-methylumbelliferyl-β-D-glucopyranoside (MUG), 4-methylumbelliferyl-β-D-cellobioside (MUC), and 4-methylumbelliferyl-β-D-xylopyranoside (MUX). Our results indicate that the snail *A. fulica* is an attractive source of cultivable bacteria that showed to be valuable resources for the production of different types of biomass-degrading enzymes.

Keywords: ***Achatina fulica*, cellulolytic bacteria, carboxymethycellulose, microbial diversity**

OPEN ACCESS

Edited by:
Gavin Collins,
National University of Ireland, Ireland

Reviewed by:
Carl James Yeoman,
Montana State University, USA
Hailan Piao,
Washington State University in Tri-Cities, USA
William James Hickey,
University of Wisconsin-Madison, USA

***Correspondence:**
Susana Frasés,
Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Avenida Carlos Chagas Filho, Centro de Ciências da Saúde - Ilha do, Fundão, 21941-902 - Rio de Janeiro, Brazil
susanafrases@biof.ufrj.br;
frases.susana@gmail.com

Specialty section:
This article was submitted to Microbiotechnology, Ecotoxicology and Bioremediation, a section of the journal Frontiers in Microbiology

Received: *31 March 2015*
Accepted: *06 August 2015*
Published: *20 August 2015*

Citation:
Pinheiro GL, Correa RF, Cunha RS, Cardoso AM, Chaia C, Clementino MM, Garcia ES, de Souza W and Frasés S (2015) Isolation of aerobic cultivable cellulolytic bacteria from different regions of the gastrointestinal tract of giant land snail Achatina fulica. Front. Microbiol. 6:860. doi: 10.3389/fmicb.2015.00860

Introduction

Cellulolytic organisms are ubiquitous in nature. Both fungi and bacteria have been heavily exploited for their abilities to produce a wide variety of cellulases and hemicellulases. Traditionally, significant emphasis has been placed on the use of fungi because they produce high amounts of extracellular enzymes, which can be easily purified and used as commercial cellulase cocktails

(Gusakov and Sinitsyn, 2012). However, novel glycoside hydrolases from bacteria have been isolated and characterized in the last few years. Bacteria have some advantages over fungi in certain aspects. In particular, they usually have a higher growth rate allowing for faster production of recombinant enzymes (Maki et al., 2009). In addition, some glycoside hydrolases from bacteria are assembled in multi-enzyme complexes that provide increased synergy, stability, and catalytic efficiency (Hou et al., 2006; Jiang et al., 2006; Waeonukul et al., 2009), while others display modular architecture (Cann et al., 1999; Zhang et al., 2014) or are multifunctional, harboring both endoglucanase and xylanase activities in the same polypeptide (Pérez-Avalos et al., 2008). Finally, cellulolytic bacteria have been isolate from harsh climate conditions (Soares et al., 2012). As consequence, their enzymes are more stable under extreme conditions (high temperature, extremes of pH) that may occur during bioconvertion processes, and this may increase the overall efficiency of the enzymatic hydrolysis and fermentation (Maki et al., 2009).

Over the years, culturable cellulolytic bacteria have been isolated from a wide variety of environments such as compost piles, decaying plant material originating from agricultural wastes, feces of ruminants, soil, gastrointestinal tract of insects, and from extreme environments such as hot springs (Doi, 2008). Screening for cellulase producing organisms may be accomplished through medium enrichment with crystalline cellulose, followed by 16S rRNA sequencing to determine the composition of the bacterial communities present and evaluate whether families containing cellulolytic species are present. Strains with cellulolytic potential can be isolated by subsequent subcultures in the enriched culture medium containing cellulose as carbon source (Maki et al., 2009; Rastogi et al., 2009). Alternatively, screening of cellulases produced by bacterial isolates may be accomplished by their cultivation in solid media containing carboxymethylcellulose (CMC) as sole carbon source, followed by Congo Red staining (Hankin and Anagnostakis, 1977). CMC is a highly specific substrate for endo-acting cellulases, as its structure has been engineered to decrystallize cellulose and create amorphous sites that are ideal for endoglucanase action, called CMCase, that cleaves intramolecular β-1,4-glucosidic bonds randomly, resulting in a dramatic reduction of the degree of polymerization and specific viscosity of CMC (Zhang et al., 2006). Although CMC has become a commonly used surrogate for cellulose, as many had associated whole cellulase activity with CMC hydrolysis (Liang et al., 2014), cellobiohydrolases are shown to be dominant in the degradation of crystalline (e.g., Avicel) and not soluble (e.g., CMC) cellulose (Zhang et al., 2006).

The giant land snail *Achatina fulica* is a terrestrial pulmonate gastropod mollusk native to East Africa that is considered an invasive pest in most of the territories in which it was introduced by human intervention. Due to its voracious appetite, great environmental adaptability, high growing and reproductive rates, this mollusk is now considered to be the most destructive terrestrial gastropod worldwide, causing ecological disequilibrium and agricultural losses (Albuquerque et al., 2008; Thiengo et al., 2008). Its success as an invasive species is mainly due to its ability to process a broad variety of vegetable organic matter. In addition to their own enzymatic repertoire, land snails contain an intriguing and adaptable microbiota that promotes the fast hydrolysis of lignocellulosic plant biomass, contributing to their impressive digestive efficiency (60–80%) (Charrier and Daguzan, 1980; Cardoso et al., 2012a). Also, recent metagenomic analysis of the crop microbiota of this snail revealed an abundance of sequences coding for oligosaccharide-degrading enzymes (36%) as well as many novel cellulose and hemicellulase coding sequences (Cardoso et al., 2012b). Although the resident bacterial diversity of *A. fulica* has been investigated recently using culture-independent molecular analysis (Pawar et al., 2012; Cardoso et al., 2012a), the cellulolytic capacity of the described bacterial communities was not assessed. Furthermore, cultivable bacteria diversity within pulmonate land snails has been partially investigated in *Helix pomatia* and *Cornu aspersum* (Charrier et al., 2006), but not been assessed in *A. fulica*. Thus, there is still need for a detailed study on the microflora from this land snail in order to identify specific bacterial isolates that are directly involved with the lignocellulosic biomass degradation.

The main focus of this work was to isolate cultivable CMC-degrading bacteria from the digestive tract of *A. fulica* in order to evaluate the biotechnological potential for their secreted hydrolytic enzymes. We were able to obtain 40 bacterial isolates, which were identified by 16S rRNA gene sequencing and additionally evaluated by phenotypic characterization using biochemical markers. The hydrolytic repertoire of the strains was investigated by enzymatic plate assays, using distinct substrates. This is the first study that focused on the evaluation of cellulolytic bacterial communities resident in *A. fulica*, showing that this land snail is a valuable source of bacterial species that can be cultivated to produce different types of cellulases.

Materials and Methods

Sampling

Three field-collected *A. fulica* snails weighing in the range of 70–80 g were captured in Rio de Janeiro, Brazil. To minimize the occurrence of transient bacteria within digestives fluids, the snails were kept inside plastic boxes (40 cm long, 20 cm wide, 20 cm high) under starvation conditions, without water and or other substrates for 24 h after capture before sample collection.

Recovery of Bacteria from Crop, Intestine, and Rectum Luminal Fluids

The snails were anesthetized according to Chung (1985), by injecting the pallial cavity with 0.5 mL of 0.01% succinylcholine chloride in 2% $MgCl_2$ solution and immediately dissected inside a biosafety cabinet. Digestive tubes were placed in a sterile Petri dish covered with a wax layer and three segments were isolated per snail: the first digestive cavity, the crop (C), that contains great amounts of a red viscous digestive fluids; the intestine (I), which comprises the proximal intestine (PI), embebed within the digestive gland and the uncovered distal intestine (DI); and the rectum (R), which is the last digestive section (Charrier and Brune, 2003). The selected segments were opened using a sterile blade and luminal contents from the same segment were pooled

and suspended vigorously in a 15-ml Falcon tube containing 10 ml of PBS (phosphate-buffered saline) (8 g.l^{-1} NaCl; 0.2 g.l^{-1} KCl; 1.44 g.l^{-1} $Na_2HPO_4{\cdot}12H_2O$; 0.24 g.l^{-1} KH_2PO_4; pH 7.6). Then, the suspension was centrifuged at 5000 × g for 15 min. Supernatant was discarded to remove endogenous cellulases and the pellet was washed twice in 10 mL of sterile PBS. Finally, pellets were suspended in 1 ml of sterile PBS.

Endoglucanase Activity as First Selection Pressure (CMCase Activity)

The three luminal suspensions (crop, intestine, and rectum) were serially diluted in PBS, ranging from 10^{-3} to 10^{-5}, and plated in triplicate onto solid minimal media (MM) containing carboxymethylcellulose (CMC) (carboxymethylcellulose sodium salt, low viscosity, from Sigma Aldrich) as the sole carbon source [CMC media: 5 g.l^{-1} CMC; 20 g.l^{-1}agar; 6.8 g.l^{-1} Na_2HPO_4; 3 g.l^{-1}KH_2PO_4; 0.5 g.l^{-1} NaCl; 1.3 g.l^{-1} $(NH_4)_2SO_4$ and 0.5 g.l^{-1} $MgSO_4.7H_2O$]. Plates were incubated for 3 days at 30°C and the resulting discrete colonies were picked and streaked four times onto new CMC plates to insure they could utilize CMC as the sole carbon source and were not using residual nutrients from the intestinal fluids (Robson and Chambliss, 1989). Pure isolates were subjected to Congo red staining (Teather and Wood, 1982). Strains were designated C to indicate isolation from the crop; I, from intestine; R, from rectum. The use of Congo-Red as an indicator for CMC degradation in an agar medium provides the basis for a rapid and sensitive first screening test for cellulolytic microbes. Isolates were maintained on CMC plates for additional experiments and also stored in 15% glycerol at −80°C for future use. After this first screening, isolates were analyzed for their capacity to degrade other polysaccharides.

Enzymatic Plate Assay

In order to evaluate the repertoire of secreted hydrolytic enzymes, the isolates were grown on MM plates containing distinct substrates: 1 mM pNPC (p-nitrophenyl-β-D-cellobioside) (Deshpande et al., 1984); 0.04% MUC (4-methylumbelliferyl-β-D-cellobioside) (Heptinstall et al., 1986); 0.04% MUG (4-methylumbelliferyl-β-D-glucopyranoside) (Heptinstall et al., 1986); 1 mM pNPG (p-nitrophenyl-β-D-glucopyranoside) (Deshpande et al., 1984); 0.04% MUX (4-methylumbelliferyl-β-D-xylopyranoside) (Bruyne and Loontiens, 1965) or 0.5% powdered sugarcane bagasse (Lucena et al., 2011) as carbon sources. Plates were incubated at 30°C for 3 days before enzyme detection.

Visualization of Enzymatic Activity

For bagasse and CMC substrates, enzyme detection was based on the appearance of a clearance halo surrounding the colonies after Congo red staining (Robson and Chambliss, 1989). Colonies harboring negative halos up to 2 mm wide relative to the colony boundary were classified as positive (+) for the CMCase secretion and those that showed halos greater than 2 mm were classified as double positive (++). For the fluorescent substrates MUC, MUG, and MUX, plates were examined under UV light (302 nm) on a Gel Doc XR+ Imaging System (Bio-Rad, Hercules, USA). Pictures were taken with the Image Lab 2.0 Software (Bio-Rad) using the automatic exposure time mode. Colonies harboring fluorescent halos were classified as positive (+) when the halos could only be visualized using the exposure time optimized for faint bands (high exposure time). Colonies whose fluorescent halos were detected upon intense bands exposure time mode (low exposure time) were categorized as double positive (++) for the respective enzyme secretion. For the colorimetric substrates pNPC and pNPG, the enzyme secretion was proportional to the development of yellow colored halos surrounding the colonies. Colonies harboring colored halos up to 5 mm wide relative to the colony boundary were classified as positive (+) and those who showed halos greater than 5 mm were classified as double positive (++) for the enzyme secretion.

Phenotypic Bacterial Characterization

For preliminary morphological characterization isolates Gram stain was performed and evaluated by light microscopy. Cultures were grown in liquid CMC medium at 30°C for 2 days at 150 rpm. After, cells were washed with sterile PBS and 5 uL of the suspension were transferred to glass slides, heat-fixed and stained according to Gram's procedure (Holt et al., 1994). The stained slides were imaged under a Leica DM 5000B microscope (Leica Microsystems, Buffalo Grove, IL, USA). Pictures were taken at 100X magnification using Leica Application Suite Software (Leica Microssystems).

Biochemical Characterization

Bacterial isolates were analyzed by biochemical tests measuring carbon source utilization, enzymatic activities, and antibiotic resistance using Vitek2 identification System (BioMérieux, Marcy l'Étoile, France), according to the manufacturer's recommendations. Briefly, bacterial suspensions turbidity was adjusted to 0.5 McFarland standard in 0.45% sodium chloride. Then, GN (Gram negative), GP (Gram positive) cards, and bacterial suspensions were manually loaded into the Vitek2 System.

The GN identification card includes tests for the following reactions: beta-galactosidase, beta-N-acetyl-glucosaminidase, glutamyl-arylamidase-pNAl, gamma-glutamyl-transferase, beta-glucosidase, beta-xylosidase, beta-alanine-arylamidase-pNA, alpha-glucosidase, beta-N-acetyl-galactosaminidase, alpha-galactosidase, phosphatase, glycine-arylamidase, beta-glucuronidase, glu-gly-arg-arylamidase, ala-phe-pro-arylamidase, L-pyrrolydonyl-arylamidase, L-proline-arylamidase, lípase, tyrosine-arylamidase, urease, ornithine-decarboxylase, lysine-decarboxylase, fermentation of glucose, H2S-production, and Ellman's test. The GN card also tests acid production from the following substrates: sucrose, glucose, adonitol, arabitol, cellobiose, maltose, mannitol, mannose, palatinose, sorbitol, trehalose, and tagatose. Finally, the following tests are also included: assimilation of malate, lactate, citrate, malonate, 5-keto-D-gluconate, coumarate, and histidine, as well as alkalinization of succinate and lactate.

The GP identification card includes test for the following reactions: phosphatidylinositol phospholipase C, arginine dihydrolase (two tests), β-galactosidase, α-glucosidase, alanine-phenylalanine-proline arylamidase, L-aspartate arylamidase,

β-galactosidase, α-mannosidase, alkaline phosphatase, l-leucine arylamidase, proline arylamidase, β-glucuronidase (two tests), α-galactosidase, L-pyrrolidonyl-arylamidase, alanine arylamidase, tyrosine arylamidase and urease. The GP identification card also tests acid production from the following substrates: amygdalin, xylose, α-cyclodextrin, sorbitol, galactose, ribose, lactate, lactose, N-acetyl-glucosamine, maltose, mannitol, mannose, methyl-β-d-glucopyranoside, pullulan, raffinose, salicin, sucrose, and trehalose. Finally, growth in 6.5% NaCl as well as tests for resistance to polymyxin B, bacitracin, novobiocin, O129, and optochin are also included in the GP identification card.

16S rRNA Gene Cloning and Sequencing

Bacterial genomic DNA was extracted using the "GenElute Bacterial Genomic DNA" kit (Sigma-Aldrich; St. Louis, USA) according to manufacturer's instructions and PCR-amplified with the universal bacterial primers 27BF (5′-*AGAGTTTGATCCTGGCTCAG*-3′) and 907RAB (5′-*TTTGAGTTTMCTTAACTGCC*-3′) for the 16S rRNA gene (Weisburg et al., 1991), using the following conditions: 5 min hot start at 94°C, followed by denaturation for 60 s at 94°C, annealing for 30 s at 54°C and 60 s of extension at 72°C. On the 35th and final cycle, the extension time was increased to 7 min. PCR products were purified using the "QIAquick PCR purification Kit" (Qiagen; Hilden, Germany) following the manufacturer's instructions. Sequencing was bidirectional (primers 27BF and 907RAB) and was performed using the MegaBace1000 DNA analysis system (GE Healthcare; Buckinghamshire, UK). Partial 16S rRNA sequences generated in this study have been deposited in GenBank under the accession numbers sequentially numbered from KF530754 (C1) to KF530793 (R40.2). Supplementary Table 1S lists the correspondence of isolate ID to Genbank accession.

Bioinformatic Analysis

Sequence assemblies were obtained with the CAP3 Assembly Program (http://pbil.univ-lyon1.fr/cap3.php) and searches against GenBank non-redundant databases were performed using the Basic Local Alignment Search Tool (BLAST) algorithm (Altschul et al., 1990). Alignments with representative bacterial sequences obtained at GenBank databases were carried out using MUSCLE software (Edgar, 2004). Phylogenetic analyses were carried out with MEGA software (Tamura et al., 2011) and the tree was constructed by neighbor-joining algorithm based on distance estimates calculated by the Kimura-2 parameter which includes a bootstrap test with 1000 replicates.

Results

Isolation of Cellulolytic Aerobic Bacteria from Crop, Intestine, and Rectum Luminal Fluids from the Giant Snail *Achatina Fulica*

In this work, we investigated the composition of the cultivable CMC-degrading bacterial community in three parts of the digestive tract of the land snail *A. fulica*: crop (C), intestine (I), and the rectum (R). In our screening, a total of 40 CMC-degrading isolates were obtained from all combined tested snail samples. For the preliminary evaluation of the cellulolytic activity, we performed the Congo red staining method on CMC agar plates to identify the CMCase-secreting isolates (**Figure 1**). Of the 40 isolates able to grown on CMC plates, a total of 24 bacterial isolates hydrolyzed CMC. Sixteen isolates were able to grew on CMC as their sole carbon sources, but did not display visible degradation halos for CMCase (**Table 4**).

Characterization of Bacterial Isolates by Phylogenetic and Enzymatic Analysis

The DNA fragments for 16S rRNA genes of the 40 isolates were amplified and sequenced. The resulting sequences were subjected to Blast analysis against GenBank non-redundant databases. The Blast matching with the highest score is shown for each isolate (**Table 1**). Our 40 isolates belonged to three bacterial phyla, namely *Proteobacteria* (24 isolates),

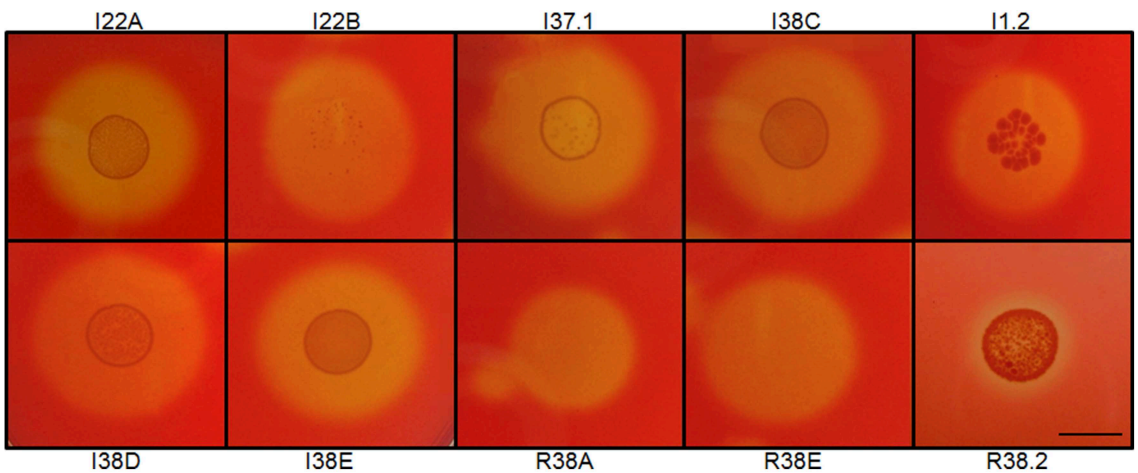


FIGURE 1 | Examples of Congo red staining of cellulolytic aerobic bacteria from the gastrointestinal lumen of the giant snail *Achatina fulica*. Strains were designated as C to indicate isolation from the crop; I, from intestine; R, from rectum. Numerals indicated clone number. Scale bar, 1.0 cm.

TABLE 1 | Molecular identification of the isolates.

Isolate	16S identification	Gram	Accession	Genbank description	Score	Coverage (%)	Identity (%)	Phylum
C1	*Pseudomonas*	–	AB681730.1	*Pseudomonas nitroreducens* subsp. *thermotolerans* gene for 16S rRNA, partial cds.	1487	99	99	*Proteobacteria*
C2	*Klebsiella*	–	AB680060.1	*Klebsiella pneumoniae* gene for 16S rRNA, partial sequence, strain: NBRC 3318	1395	100	98	*Proteobacteria*
C3	*Sphingobacterium*	–	NR_042134.1	*Sphingobacterium mizutaii* strain DSM 11724 16S ribosomal RNA, partial sequence	1014	100	99	*Bacteroidetes*
C5	*Paracoccus*	–	AB680283.1	*Paracoccus denitrificans* gene for 16S rRNA, partial sequence, strain: NBRC 12442	1371	99	100	*Proteobacteria*
C6	*Paracoccus*	–	JQ321836.1	*Paracoccus* sp. YF1 16S ribosomal RNA gene, partial sequence	1266	99	100	*Proteobacteria*
C7	*Sphingobacterium*	–	FJ459994.1	*Sphingobacterium multivorum* 16S ribosomal RNA gene, partial sequence	1485	99	99	*Bacteroidetes*
C8	*Microbacterium*	+	AB646581.2	*Microbacterium* sp. SL10 gene for 16S ribosomal RNA, partial sequence	1256	99	96	*Actinobacteria*
C9	*Pseudomonas*	–	AB646255.1	*Pseudomonas* sp. H-8-1-3 gene for 16S ribosomal RNA, partial sequence	1500	100	99	*Proteobacteria*
C10	*Flavobacterium*	–	DQ168834.1	Uncultured *Flavobacterium* sp. clone J16 16S ribosomal RNA gene, partial sequence	1432	100	98	*Bacteroidetes*
C11	*Aeromonas*	–	NR_029252.1	*Aeromonas punctata* strain ATCC 15468 16S ribosomal RNA, partial sequence	1515	100	99	*Proteobacteria*
C12	*Aeromonas*	–	NR_029252.1	*Aeromonas punctata* strain ATCC 15468 16S ribosomal RNA, partial sequence	1391	100	96	*Proteobacteria*
C13.4	*Aeromonas*	–	NR_029252.1	*Aeromonas punctata* strain ATCC 15468 16S ribosomal RNA, partial sequence	1330	100	98	*Proteobacteria*
C14	*Pseudomonas*	–	GU979230.1	*Pseudomonas* sp. WP6 16S ribosomal RNA gene, partial sequence	1426	100	99	*Proteobacteria*
C15	*Aeromonas*	–	NR_029252.1	*Aeromonas punctata* strain ATCC 15468 16S ribosomal RNA, partial sequence	1068	96	97	*Proteobacteria*
C16	*Microbacterium*	+	AB646581.2	*Microbacterium* sp. SL10 gene for 16S ribosomal RNA, partial sequence	1482	100	98	*Actinobacteria*
C18	*Pseudomonas*	–	JQ701740.1	*Pseudomonas putida* strain jvu23 16S ribosomal RNA gene, partial sequence	1423	99	99	*Proteobacteria*
C19	*Aeromonas*	–	NR_029252.1	*Aeromonas punctata* strain ATCC 15468 16S ribosomal RNA, partial sequence	1465	98	98	*Proteobacteria*
C20	*Pseudomonas*	–	AB513735.1	*Pseudomonas putida* gene for 16S ribosomal RNA, partial sequence, strain: 1106	1439	99	98	*Proteobacteria*
C21.1	*Aeromonas*	–	NR_029252.1	*Aeromonas punctata* strain ATCC 15468 16S ribosomal RNA, partial sequence	1461	99	99	*Proteobacteria*
C22	*Aeromonas*	–	NR_029252.1	*Aeromonas punctata* strain ATCC 15468 16S ribosomal RNA, partial sequence	1504	99	99	*Proteobacteria*
C23	*Klebsiella*	–	NR_025635.1	*Klebsiella variicola* strain F2R9 16S ribosomal RNA, partial sequence	1450	99	98	*Proteobacteria*
C24.1	*Aeromonas*	–	AB626132.1	*Aeromonas* caviae gene for 16S rRNA, partial sequence, strain: JCM 1060	1456	100	98	*Proteobacteria*
C24.2	*Paracoccus*	–	NR_026457.1	*Paracoccus pantotrophus* strain ATCC 35512 16S ribosomal RNA, partial sequence	976	98	99	*Proteobacteria*
C25	*Aeromonas*	–	JF920485.1	*Aeromonas caviae* strain E4EL26 16S ribosomal RNA gene, partial sequence	1506	98	99	*Proteobacteria*
I 1.2	*Streptomyces*	+	NR_043823.1	*Streptomyces kunmingensis* strain NRRL B-16240 16S ribosomal RNA, partial sequence	1450	100	99	*Actinobacteria*
I22A	*Cellulosimicrobium*	+	AB188217.1	*Cellulosimicrobium* sp. TUT1222 gene for 16S rRNA, partial sequence	1421	100	99	*Actinobacteria*
I22B	*Cellulosimicrobium*	+	JQ659848.1	*Cellulosimicrobium funkei* strain R6-417 16S ribosomal RNA gene, partial sequence	1480	99	99	*Actinobacteria*
I28A	*Klebsiella*	–	AB114637.1	*Klebsiella* sp. PN2 gene for 16S rRNA	1443	97	99	*Proteobacteria*

(Continued)

TABLE 1 | Continued

Isolate	16S identification	Gram	Accession	Genbank description	Score	Coverage (%)	Identity (%)	Phylum
I32.1	*Enterobacter*	–	JQ396391.1	*Enterobacter* sp. PXG11 16S ribosomal RNA gene, partial sequence	1256	99	99	*Proteobacteria*
I32.2	*Stenotrophomonas*	–	DQ242478.1	*Stenotrophomonas* sp. D-A 16S ribosomal RNA gene	1361	88	100	*Proteobacteria*
I37.1	*Cellulosimicrobium*	+	AB166888.1	*Cellulosimicrobium* cellulans gene for 16S rRNA, partial sequence	1441	99	99	*Actinobacteria*
I38C	*Cellulosimicrobium*	+	JQ659856.1	*Cellulosimicrobium* funkei strain R6-437 16S ribosomal RNA gene, partial sequence	1472	99	99	*Actinobacteria*
I38D	*Cellulosimicrobium*	+	HM367604.1	*Cellulosimicrobium* sp. GE2 16S ribosomal RNA gene, partial sequence	1375	100	98	*Actinobacteria*
I38E	*Cellulosimicrobium*	+	JQ659856.1	*Cellulosimicrobium* funkei strain R6-437 16S ribosomal RNA gene, partial sequence	1384	100	97	*Actinobacteria*
R7.1	*Agromyces*	+	NR_043931.1	*Agromyces allii* strain UMS-62 16S ribosomal RNA, partial sequence	1365	100	97	*Actinobacteria*
R38.2	*Nocardiopsis*	+	HQ433551.1	*Nocardiopsis* sp. KNU 16S ribosomal RNA gene, partial sequence	1482	99	99	*Actinobacteria*
R38A	*Microbacterium*	+	JQ659823.1	*Microbacterium binotii* strain R6-367 16S ribosomal RNA gene	1411	100	99	*Actinobacteria*
R38-E1	*Microbacterium*	+	JQ659823.1	*Microbacterium binotii* strain R6-367 16S ribosomal RNA gene, partial sequence	1362	99	100	*Actinobacteria*
R40.1	*Klebsiella*	–	JQ305691.1	*Klebsiella variicola* strain ISB-6 16S ribosomal RNA gene, partial sequence	1424	99	99	*Proteobacteria*
R40.2	*Pseudomonas*	–	AB681703.1	*Pseudomonas putida* gene for 16S rRNA, partial sequence, strain: NBRC 102092	1476	99	99	*Proteobacteria*

Actinobacteria (13 isolates), and *Firmicutes* (3 isolates). These 40 isolates showed their closest matches to 13 distinct genera, 6 of the *Proteobacteria* phyla (*Aeromonas*, *Pseudomonas*, *Klebsiella*, *Enterobacter*, *Stenotrophomonas,* and *Paracoccus*), 5 of *Actinobacteria* (*Streptomyces*, *Cellulosimicrobium*, *Agromyces*, *Microbacterium,* and *Nocardiopsis*), and 2 of *Bacteroidetes* (*Sphingobacterium* and *Flavobacterium*). In the crop, members of the genera *Aeromonas* were the most predominant and accounted for 38% of total numbers of isolates identified. In intestine, species of the genera *Cellulosimicrobium* spp. were the most predominant (60% of total intestine isolates). Interestingly, all of the representatives of the *Aeromonas* genera identified in our study were recovered exclusively from the crop fluids, whereas species of *Cellulosimicrobium* were recovered only from intestine. In the rectum, there was not a predominance of any cellulolytic isolate over the others.

Phylogenetic relationships of the isolates together with representative 16S bacterial sequences were also analyzed (**Figure 2**). In order to identify the phylogenetic groups that were most efficient in degrading cellulosic compounds, their general repertoire of oligosaccharide-degrading enzymes were evaluated in parallel by enzymatic plate assays (**Figure 3**). The isolates were ordered by hydrolysis profile similarities and a summary is shown in **Table 2**. The resulting tree showed that the 40 isolates could be classified into several groups on the basis of similarities in 16S rRNA sequences. Notably, similar hydrolytic profiles could be visualized among phylogenetic-related isolates (**Table 2**). In the *Cellulosimicrobium* branch, the isolates I22A, I22B, and I37.1 were closely related to *Cellulosimicrobium funkei*, whereas I38C and I38D were more related to *Cellulosimicrobium cellulans* (**Figure 2**). I38E was put in a separate branch of the tree and showed only 97% of identity with *C. funkei* 16S rRNA sequence (**Table 1**). Four isolates were grouped in the *Microbacterium* branch. R38A and R38E were closely related to *Microbacterium binotii* (100 and 99% identity, respectively), whereas C8 and C16 were related, in a separate branch and to a lesser extent, to *Microbacterium paraoxydans* (96 and 98% identity, respectively) (**Figure 2**). This phylogenetic separation between R38A/R38E and C8/C16 agreed well with their cellulolytic potentials. Whereas, R38A and R38E were highly cellulolytic, as showed by the enzymatic plate assay, C8 and C16 were not capable of hydrolyzing the sugarcane bagasse or CMC, only the cello-oligosaccharides MUG, pNPG, and MUC (**Table 2**). The isolate R7.1 showed 97% identity with *Agromyces allii* strain UMS-62 16S rRNA sequence (NR_043931.1) (**Table 1**). Although many members of the genus *Agromyces* have been isolated worldwide from soil (Li et al., 2003; Jurado et al., 2005; Yoon et al., 2008; Zhang et al., 2010), their cellulolytic capacities were not reported. The isolates R7.1, R38.2, R38A, R38E, I22A, I22B, I37.1, I38C, I38D and I38E, all from *Actinobacteria* phylum, displayed very similar hydrolytic profiles (**Table 2**), being able to degrade all of the substrate tested, including the highly recalcitrant powdered sugarcane bagasse. Interestingly, all of the bagasse-degrading isolates also hydrolyzed CMC. All of the CMC- and bagasse-degrading isolates also degrade pNPG and MUG, however, five isolates (C3, C8, C10, C16, I28A,

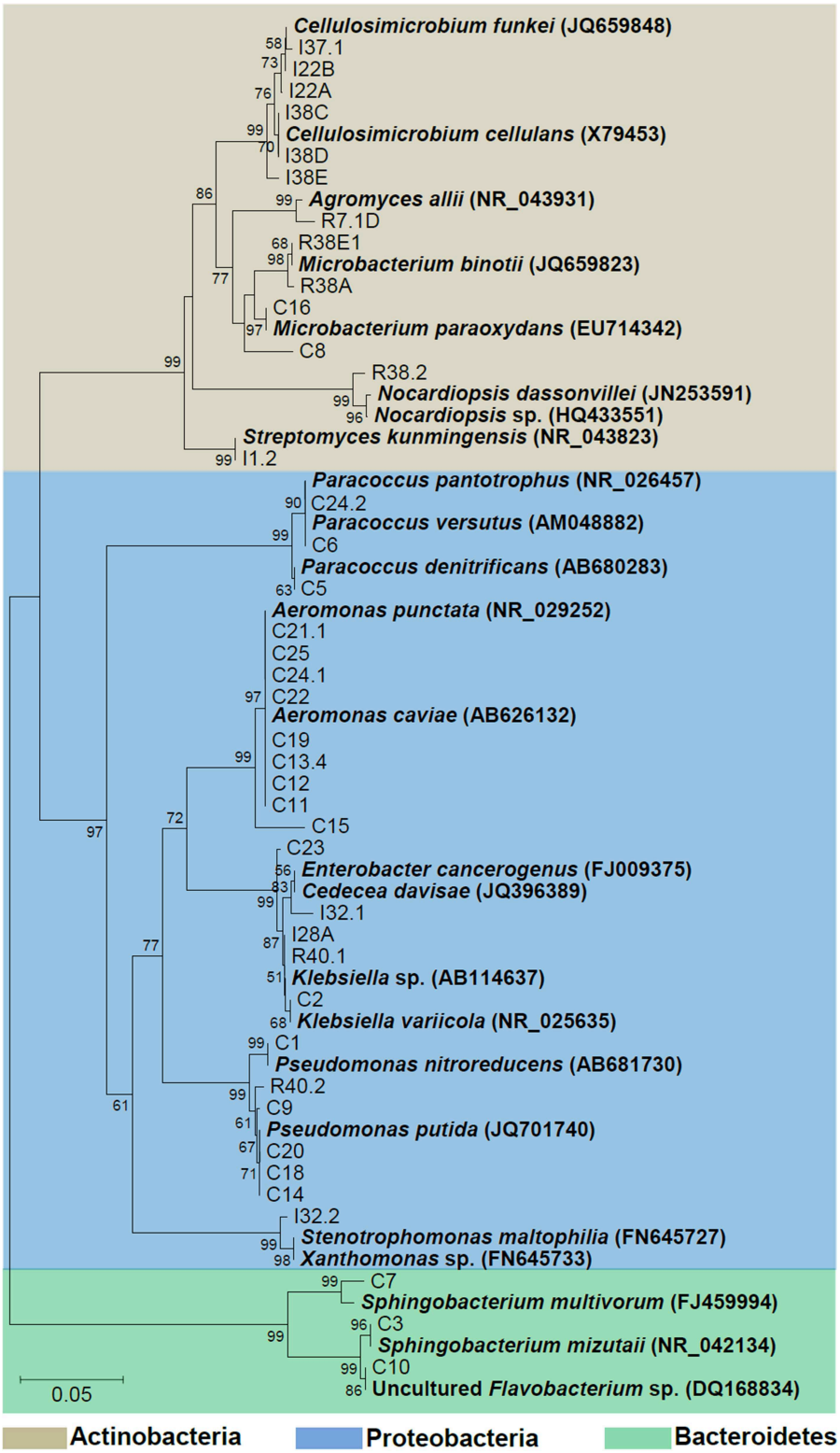


FIGURE 2 | Phylogenetic tree of isolates. The 16S sequences of the isolated bacteria are aligned with reference strains. Reference bacterial 16S sequences from GenBank are in bold. Alignments with representative bacterial sequences obtained at GenBank databases were carried out using MUSCLE. Phylogenetic analyses were carried out with MEGA and tree was constructed by neighbor-joining algorithm based on distance estimates calculated by the Kimura-2 parameter which includes a bootstrap test with 1000 replicates. Strains were designated C to indicate isolated from crop; I, from intestine; R, from rectum.

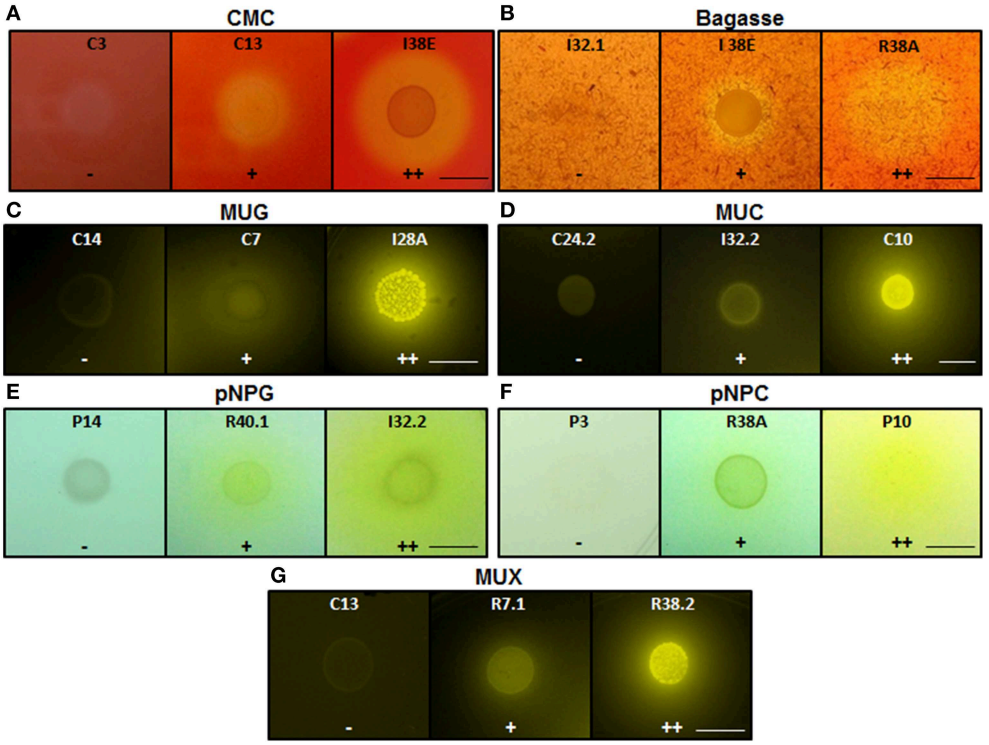


FIGURE 3 | Enzymatic agar plate assay. Representative negative, positive, and double positive isolates for each substrate are shown. **(A)** CMC, carboxymethylcellulose; **(B)** Bagasse, powdered sugarcane bagasse; **(C)** MUG, 4-methylumbelliferyl-β-D-glucopyranoside; **(D)** MUC, 4-methylumbelliferyl-β-D-cellobioside; **(E)** pNPG, p-nitrophenyl-β-D-glucopyranoside; **(F)** pNPC, p-nitrophenyl-β-D-cellobioside; and **(G)** MUX, 4-methylumbelliferyl-β-D-xylopyranoside. For bagasse and CMC, the enzyme detection was based on the appearance of negative halo after Congo red stain. For the fluorescent MUC, MUG, and MUX, the plates were UV-irradiated. For the colorimetric substrates pNPC and pNPG, the enzymatic activity was proportional to the development of yellow color. Legends: (–), no detectable hydrolysis; (+), hydrolysis; (++), high hydrolysis. Strains were designated C to indicate isolated from crop; I, from intestine; R, from rectum. Scale bar, 1.0 cm. Note that scale bar applies to all three panels in a series. Also note that strain IDs are shown.

I32.1, I32.2) that secrete β-glucosidase didn't degrade CMC or bagasse.

The isolates C11, C12, C13.4, C15, C19, C21.1, C22, C24.1, and C25 were all related to *Aeromonas punctata* and *Aeromonas caviae* (**Figure 2**) and all of them were capable of hydrolyzing both CMC and the recalcitrant sugarcane bagasse (**Table 2**), making them promising tools for the discovery of novel cellulases. The isolates C2, C23, I28A, I32.1, and R40.1 were grouped in the *Klebsiella/Enterobacter* branch. Interestingly, these five isolates showed very similar cellulolytic patterns, being able to hydrolyze CMC (except I28 and I32.1), MUG, MUC, and MUX, but not sugarcane bagasse (**Table 2**). Six isolates were grouped in the *Pseudomonas* branch. C1 was closely related to *Pseudomonas nitroreducens* (99% identity), while C9, C14, C18, C20, and R40.2 were related to *Pseudomonas putida* (98–99% identity) (**Figure 2**). The isolates C5, C6, and C24.2 were closely related to *Paracoccus denitrificans*, *Paracoccus versutus,* and *Paracoccus pantotrophus*, with 100, 99, and 99% identity, respectively. All of these isolates showed an identical hydrolytic pattern, none of them being able to secrete detectable amounts of cellulolytic enzymes (**Table 2**). The isolates C3, C7, and C10 are the only representatives of the phyla *Bacteroidetes* and were related to *Sphingobacterium mizutaii* (99% identity), *Sphingobacterium multivorum* (99% identity), and Uncultured *Flavobacterium* sp. (98% identity) (**Figure 2**). They are able to hydrolyze pNPG, MUG, pNPC, and MUC, as a consequence for their β-glucosidase secretion, but not CMC or sugarcane bagasse (**Table 2**). Interestingly, 16 isolates, mainly actinomycetes, hydrolyze the substrate MUX, specific for β-xylosidases (**Table 2**). β-xylosidases are hydrolytic enzymes which play an important role in xylan degradation, hydrolyzing xylobiose, and xylooligosaccharides from the non-reducing end to xylose. These isolates may be involved in the degradation of hemicellulose, and will be further analyzed in the future for the secretion of other enzymes such as endoxylanases.

Characterization of Bacterial Isolates by Biochemical Tests (Vitek2)

In order to confirm the taxonomic grouping based on 16S rRNA sequences, bacterial isolates were further analyzed by classical biochemical tests using automatized Vitek2 identification System. For preliminary morphological characterization and to confirm the purity of the cultures, the isolates were Gram stained and then

TABLE 2 | Enzymatic agar plate results ordered by hydrolysis profile.

Isolates	16S identification	CMC	Bagasse	MUG	p-NPG	MUC	p-NPC	MUX
C8	*Microbacterium*	–	–	+	+	+	–	–
C16	*Microbacterium*	–	–	+	+	+	–	–
R38A	*Microbacterium*	++	++	++	++	++	+	+
R38E	*Microbacterium*	++	++	++	++	++	+	+
I22A	*Cellulosimicrobium*	++	+	++	++	++	+	++
I22B	*Cellulosimicrobium*	++	+	++	++	++	+	++
I37.1	*Cellulosimicrobium*	++	+	++	++	+	+	+
I38C	*Cellulosimicrobium*	++	+	++	++	+	+	++
I38D	*Cellulosimicrobium*	++	+	++	++	+	+	++
I38E	*Cellulosimicrobium*	++	+	++	++	++	+	++
I1.2	*Streptomyces*	++	++	++	++	+	–	–
R7.1	*Agromyces*	+	+	++	++	+	+	+
R38.2	*Nocardiopsis*	+	+	+	+	+	+	+
C11	*Aeromonas*	+	+	++	++	++	+	–
C12	*Aeromonas*	+	++	++	++	+	+	–
C13.4	*Aeromonas*	+	++	++	++	+	+	–
C15	*Aeromonas*	+	++	++	++	+	+	–
C19	*Aeromonas*	+	+	++	+	++	+	–
C21.1	*Aeromonas*	+	+	++	++	+	+	–
C22	*Aeromonas*	+	+	++	++	+	+	–
C24.1	*Aeromonas*	+	–	++	++	++	+	–
C25	*Aeromonas*	+	++	++	++	+	+	–
I32.2	*Stenotrophomonas*	–	–	+	++	+	–	–
C3	*Sphingobacterium*	–	–	+	+	++	–	–
C7	*Sphingobacterium*	+	–	+	+	+	+	–
C10	*Flavobacterium*	–	–	++	++	++	++	++
C2	*Klebsiella*	+	–	++	++	+	+	++
C23	*Klebsiella*	+	–	+	++	+	+	++
I28A	*Klebsiella*	–	–	++	++	+	+	++
I32.1	*Enterobacter*	–	–	+	++	+	–	+
R40.1	*Klebsiella*	+	–	+	+	+	+	+
C1	*Pseudomonas*	–	–	–	–	–	–	–
C5	*Paracoccus*	–	–	–	–	–	–	–
C6	*Paracoccus*	–	–	–	–	–	–	–
C9	*Pseudomonas*	–	–	–	–	–	–	–
C14	*Pseudomonas*	–	–	–	–	–	–	–
C18	*Pseudomonas*	–	–	–	–	–	–	–
C20	*Pseudomonas*	–	–	–	–	–	–	–
C24.2	*Paracoccus*	–	–	–	–	–	+	–
R40.2	*Pseudomonas*	–	–	–	–	–	–	–

(–) no detectable hydrolysis; (+) hydrolysis; (++) high hydrolysis. Strains were designated C to indicate the ones isolated from crop; I, from intestine; R, from rectum.

visualized under light microscopy and photo-documented (data not shown). Thirteen isolates were found to be Gram positive and 27 Gram negative. Based on the similarities and differences in the biochemical profiles, the isolates could be assigned into six distinct groups among the Gram negative bacteria and into six distinct groups among the Gram positive isolates (**Tables 3, 4**). As expected, the biochemical profiles obtained from Vitek2 tests agreed well with the 16S taxonomic delimitation (**Figure 2**), as well with the hydrolytic profile (**Table 2**).

The Gram negative isolates C1, C9, C14, C18, C20, and R40.2 (*Pseudomonas* group, **Figure 2**) shared several biochemical traits that confirm they are all taxonomically related (**Table 3**, **Figure 2**). However, three bacterial isolates showed slight differences from each other in such biochemical properties as D-maltose utilization, presence of phosphatase and glycine arylamidase (C1 only), presence of beta-Alanine acrylamidase (C1 and R40.2), absence of urease (R40.2 only), inability to assimilate L-malate and L-lactate (R40.2), lack of malonate

TABLE 3 | Vitek2 biochemical characterization—GN Card.

TEST	Mnemonic	C1	C9	C14	C18	C20	R40.2	C2	C23	I28A	R40.1	I32.1	I32.2	C3	C7	C10	C5	C6	C24.2	C11	C12	C13.4	C15	C19	C21	C22	C24.1	C25
Ala–phe–pro–arylamidase	APPA	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	−	−	−	+	−	+	+	+	+	+	+	+
Adonitol	ADO	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	−	−	−	−	−	−	−	−	−
L–pyrrolydonyl–arylamidase	PyrA	−	−	−	−	−	−	−	+	+	+	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−
L–arabitol	lARL	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	−	−	−	−	−	−	−	−	−
D–cellobiose	dCEL	−	−	−	−	−	−	+	+	+	+	+	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+
Beta–galactosidase	BGAL	−	−	−	−	−	−	+	+	+	+	+	−	−	+	+	−	−	−	+	+	+	+	+	+	+	+	+
H2S production	H2S	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
B-N-acetylglucosaminidase	BNAG	−	−	−	−	−	−	+	+	−	+	+	−	−	+	+	−	−	−	+	+	+	+	+	+	+	+	+
Glutamyl arylamidase pNA	AGLTp	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
D–glucose	dGLU	+	+	+	+	+	+	+	+	+	+	+	−	+	−	−	−	−	−	+	+	+	+	+	+	+	+	+
Gama–glutamyl–transferase	GGT	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	−	−	−	+	−	+	−	−	+	+	−	−
Fermentation/Glucose	OFF	−	−	−	−	−	−	+	+	+	+	+	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+
Beta–glucosidase	BGLU	−	−	−	−	−	−	+	+	+	+	+	+	−	+	+	−	−	−	+	+	+	+	+	+	+	+	+
D–maltose	dMAL	+	−	−	−	−	−	+	+	+	+	+	−	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+
D–mannitol	dMAN	−	−	−	−	−	−	+	+	+	+	+	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+
D–mannose	dMNE	−	−	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Beta–xylosidase	BXYL	−	−	−	−	−	−	+	+	+	+	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−
Beta–alanine acrylamidase	BAlap	+	−	−	−	−	+	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
L–proline arylamidase	ProA	+	+	+	+	+	+	−	−	+	+	+	+	+	−	−	−	−	−	+	+	+	+	+	+	+	+	+
Lipase	LIP	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+
Palatinose	PLE	−	−	−	−	−	−	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Tyrosine arylamidase	TyrA	+	+	+	+	+	+	−	+	+	+	+	−	+	+	+	−	+	+	+	−	+	+	+	+	+	+	+
Urease	URE	+	+	+	+	+	−	−	−	+	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−
D–sorbytol	dSOR	−	−	−	−	−	−	+	+	+	+	+	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−
Saccharose/Sucrose	SAC	−	−	−	−	−	−	+	+	+	+	+	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+
D–tagatose	dTAG	−	−	−	−	−	−	−	+	−	+	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−
D–trehalose	dTRE	−	−	−	−	−	−	+	+	+	+	+	−	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+
Citrate (Sodium)	CIT	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Malonate	MNT	+	−	+	+	+	−	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
5-keto-D-gluconate	5KG	−	−	−	−	−	−	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
L–lactate alkalinisation	lLATk	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+
41 Alpha–glucosidase	AGLU	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−
Succinate alkalinisation	SUCT	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	+	−	+	+	+	+	+	+	+	+	+
B-N-acetylgalactosaminidase	NAGA	−	−	−	−	−	−	−	−	−	−	+	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−
Alpha–galactosidase	AGAL	−	−	−	−	−	−	+	+	+	+	+	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−
Phosphatase	PHOS	+	−	−	−	−	−	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
Glycine arylamidase	GlyA	+	−	−	−	−	−	−	−	+	+	+	−	−	−	+	−	−	−	−	−	+	−	−	−	+	−	−
Ornithine decarboxylase	ODC	−	−	−	−	−	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

(Continued)

TABLE 3 | Continued

TEST	Mnemonic	C1	C9	C14	C18	C20	R40.2	C2	C23	I28A	R40.1	I32.1	I32.2	C3	C7	C10	C5	C6	C24.2	C11	C12	C13.4	C15	C19	C21	C22	C24.1	C25
Lysine decarboxylase	LDC	−	−	−	−	−	−	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
L–histidine assimilation	IHISa	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Coumarate	CMT	+	+	+	+	+	+	−	−	+	−	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+
Beta–glucuronidase	BGUR	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
O/129 resistance	O129R	+	+	+	+	+	+	+	+	+	+	+	−	+	−	−	−	−	−	+	+	+	+	+	+	+	+	+
Glu–gly–arg–arylamidase	GGAA	−	−	−	−	−	−	−	−	−	−	−	+	−	+	+	−	−	−	+	−	+	+	−	+	+	+	−
L–malate assimilation	IMLTa	+	+	+	+	+	−	−	−	−	−	−	−	+	−	−	−	+	+	−	−	+	+	+	+	+	+	+
Ellman	ELLM	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+
L–lactate assimilation	ILATa	+	+	+	+	+	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−

acidification (C9 and R40.2), and inability to utilize D-mannose (C1 and C9).

The second discernible group is represented by the isolates C2, C23, I28A, I32.1, and R40.1 (*Klebsiella* group, **Figure 2**). Two isolates showed slight differences, such as presence of L-pyrrolydonyl-arylamidase (C23) and absence of tyrosine arylamidase and ornithine decarboxylase absence (C2). The isolate I32.2 displayed a biochemical pattern that was distinct from all the others, which confirms it is the unique representative of its taxonomic group, *Stenotrophomonas* (**Figure 2**). The two following groups (the one represented by C3 and C7 and the other by C5, C6, C24.2, respectively) were the most heterogeneous. This suggests that each of them constitutes distinct species from *Sphingobacterium* and *Paracoccus* genus, respectively. The last discernible group among Gram negative isolates contains C11, C12, C13.4, C15, C19, C21, C22, C24.1, and C25 (*Aeromonas* group, **Figure 2**). All were able to utilize D-cellobiose, to ferment glucose and were positive for the beta-glucosidase test (**Table 3**). Despite the overall similarities in the biochemical profile, only the isolates C24.1 and C25 were identical. The others showed slight differences, such as absence of the enzymes Ala-Phe-Pro-Arylamidase and Tyr-arylamidase (C12), presence of glycine arylamidase (C13.4 and C22), inability to assimilate L-malate (C11 and C22), and L-Lactate assimilation (C21 and C22). These slight biochemical differences suggest genetic diversity in the species level among these phenotypically related isolates.

The Gram positives isolates C8 and C16 displayed very similar biochemical profiles (**Table 4**), differing only in the D-ribose utilization and bacitracin resistance (C16 positive) and were closely related to *M. paraoxydans* (**Figure 2**). The isolates R38A and R38E displayed identical profiles and were closed related to *M. binotii* (**Table 4**, **Figure 2**). The third discernible group among Gram positives is represented by the isolates I22A, I22B, I38C, I38D, I38E, and I37.1 (*Cellulosimicrobium* group, **Figure 2**). Although I22A, I22B, and I37.1 showed very similar profiles, they are not identical, differing in the arginine dihydrolase trait (only I22A was positive), in D-ribose utilization, and presence of beta-galactosidase (I37.1 only). Likewise, the closely related I38C, I38D, and I38E, differ from I22 by the presence of beta-galactopiranosidase, L-lactate alkalinisation, and novobiocin resistance. Differences in these three isolates, however, occur in the Salicin and O/129 resistance (**Table 4**). Finally, each of the isolates I1.2, R7.1, and R38.2 displayed an unique biochemical pattern, which confirms they are the sole representatives of their taxonomic group *Streptomyces*, *Agromyces,* and *Nocardiopsis*, respectively (**Figure 2**).

Discussion

The land snail *A. fulica* is a voracious herbivorous with great environmental and ecological importance. Most of its capacity to process a broad variety of vegetable organic matter is due to the presence of cellulolytic enzymes, both from the animal and resident microbiota. The bacterial communities inside the gut of this snail may have crucial importance in cellulose and other plant wall components digestion. As the

TABLE 4 | Vitek 2 biochemical characterization—GP Card.

TEST	Mnemonic	C8	C16	R38A	R38E	I22A	I22B	I38C	I38D	I38E	I37.1	I1.2	R7.1	R38.2
D-amygdalin	AMY	–	–	–	–	–	–	–	–	–	–	–	–	+
Phosphatidylinositolphospholip	PIPLC	–	–	–	–	–	–	–	–	–	–	–	–	–
D-xylose	dXYL	–	–	+	+	+	+	+	+	+	+	–	+	–
Arginine dihydrolase	ADH1	+	+	+	+	+	–	–	–	–	–	–	+	–
Beta-galactosidase	BGAL	+	+	+	+	–	–	+	+	+	+	–	+	–
Alfa-glucosidase	AGLU	+	+	+	+	+	+	+	+	+	+	+	+	+
Ala-phe-pro arylamidase	APPA	+	+	+	+	+	+	+	+	+	+	+	–	+
Cyclodextrin	CDEX	–	–	–	–	–	–	–	–	–	–	–	–	–
L-aspartate arylamidase	AspA	–	–	–	–	–	–	–	–	–	–	–	–	–
Beta galactopyranosidase	BGAR	–	–	–	–	–	–	+	+	+	–	–	+	–
Alfa-mannosidase	AMAN	+	+	–	–	–	–	–	–	–	–	–	–	–
Alkaline phosphatase	PHOS	–	–	–	–	–	–	–	–	–	–	–	–	–
Leucine arylamidase	LeuA	+	+	+	+	+	+	+	+	+	+	+	+	+
L-Proline arylamidase	ProA	+	+	+	+	+	+	+	+	+	+	+	+	+
Beta glucuronidase	BGURr	–	–	–	–	–	–	–	–	–	–	–	–	+
Alpha-galactosidase	AGAL	–	–	+	+	–	–	–	–	–	–	–	–	+
L-Pyrrolidonyl-arylamidase	PyrA	–	–	–	–	+	+	+	+	+	+	–	–	–
Beta glucuronidase	BGUR	–	–	–	–	–	–	–	–	–	–	–	–	–
Alanine arylamidase	ALaA	+	+	+	+	+	+	+	+	+	+	+	+	+
Tyrosine arylamidase	TyrA	+	+	+	+	+	+	+	+	+	+	–	+	+
D-sorbitol	dSOR	–	–	–	–	–	–	–	–	–	–	–	–	–
Urease	URE	–	–	–	–	–	–	–	–	–	–	+	–	+
Polymixin B resistance	POLYB	–	–	–	–	–	–	–	–	–	–	–	–	–
D-galactose	dGAL	+	+	+	+	+	+	+	+	+	+	+	+	–
D-RiBOSE	dRIB	–	+	–	–	–	–	+	+	+	+	–	–	–
L-lactate alkalinisation	lLATk	+	+	+	+	–	–	+	+	+	–	–	–	–
Lactose	LAC	–	–	–	–	–	–	–	–	–	–	–	–	–
N-acetyl-D-glucosamine	NAG	–	–	–	–	+	+	+	+	+	+	–	–	–
D-maltose	dMAL	+	+	+	+	+	+	+	+	+	+	–	+	–
Bacitracin resistance	BACI	–	+	–	–	–	–	–	–	–	–	–	–	–
Novobiocin resistance	NOVO	–	–	–	–	–	–	+	+	+	–	–	–	–
Growth in 6.5% NaCl	NC6.5	–	–	–	–	–	–	–	–	–	–	–	–	–
D-mannitol	dMAN	+	+	+	+	–	–	–	–	–	–	–	–	–
D-mannose	dMNE	+	+	+	+	+	+	+	+	+	+	–	+	–
Methyl-B-D-glucopyranoside	MBdG	–	–	+	+	–	–	–	–	–	–	–	–	–
Pullulan	PUL	–	–	–	–	–	–	–	–	–	–	–	–	–
D-raffinose	dRAF	–	–	–	–	–	–	–	–	–	–	–	–	–
O/129 resistance (comp. Vibrio.)	O129R	–	–	–	–	–	–	–	+	+	–	–	–	–
Salicin	SAL	–	–	+	+	–	–	–	–	+	–	–	+	–
Saccharose/Sucrose	SAC	+	+	+	+	+	+	+	+	+	+	+	–	–
D-treahlose	dTRE	+	+	+	+	+	+	+	+	+	+	–	–	–
Arginine dihydrolase 2	ADH2s	–	–	–	–	–	–	–	–	–	–	–	–	+
Optochin resistance	OPTO	–	–	+	+	+	+	+	+	+	+	–	–	–

first steps of this study, we assumed that the different gut regions such as the crop, intestine and rectum are highly specialized compartments, and each could have a distinct role to play in digestion, as well as particular resident microbial communities. Based on the 16S rRNA gene sequence, our 40 isolates showed their closest matches to 13 distinct genera, six of the *Proteobacteria* phyla (*Aeromonas*, *Pseudomonas*, *Klebsiella*, *Enterobacter*, *Stenotrophomonas,* and *Paracoccus*), five of *Actinobacteria* (*Streptomyces*, *Cellulosimicrobium*, *Agromyces*, *Microbacterium,* and *Nocardiopsis*), and two of *Bacteroidetes* (*Sphingobacterium* and *Flavobacterium*). Although we have selected exclusively cultivable CMC-degrading bacterial species in our screening method, many of the genera identified in this work were reported in previous studies based on metagenomic

approaches (Cardoso et al., 2012a,b), which per definition detect also non-cultivable, anaerobic, and non-cellulolytic species. For instance, Cardoso et al. (2012a) identified the following bacterial taxa whose representatives were also isolated in our study: *Enterobacter* (24 clones) *Klebsiella* (16 clones), *Aeromonas* (89 clones, 87 from crop fluid), *Pseudomonas* (38 clones), *Xanthomonas* (48 clones), *Microbacterium* (4 clones, exclusively from rectum), and *Flavobacterium* (25 clones). Similar to our findings, Cardoso et al. (2012a) showed that the bacterial community structure of crop fluid was different from that of the feces (named rectum, in our study), suggesting that this land snail microbiota changes according to the gut region. Besides the above mentioned species, they were able to detect representative 16S rRNA sequences of the following taxa, which were not isolated in our study: *Sulfurospirillum* (72 clones), *Citrobacter* (39 clones) *Clostridiaceae* (47 clones), *Lactococcus* (44 clones), and *Mucilaginibacter* (70 clones). The reasons that could account for the absence of these taxa in our screening is the need for anaerobic or microaerophilic conditions for growth, in the case of *Clostridiaceae* (Ko et al., 2011) and *Sulfurospirillum* (Lancaster and Simon, 2002; Luijten et al., 2003); the inability to degrade CMC, in the case of non-cellulolytic species of the *Sulfurospirilum* genus (Pankratov et al., 2007), and the natural shift in gut microbial communities that takes place in snails according to their diet (Cardoso et al., 2012a). Likewise, in an independent metagenomic analysis of the microbiota from the crop of *A. fulica* (Cardoso et al., 2012b), the genera *Pseudomonas* (37.5%), *Sulfurospirillum* (8.5%), and *Stenotrophomonas* (7.3%) were assigned as principal bacterial groups.

The 16S rRNA based taxonomic delimitation was corroborated by our biochemical profiling using the Vitek cards. Based on similarities and differences on the biochemical profile, the isolates could be assigned into 12 distinct groups (**Tables 3, 4**) whose component isolates are grouped in a very similar way in the phylogenetic tree (**Figure 2**). Besides, it is noteworthy that all of these phenotypic-related isolates belong to the same cellulolytic profile group (**Table 2**), indicating a clear correlation between molecular taxonomy, biochemical profile, and cellulolytic phenotype.

The isolates C14, C18, and C20 exhibited the same biochemical Vitek profile and were placed in the same cluster (together with *P. putida*) in the 16S rRNA gene tree, with zero distance (**Figure 2**). This which may indicate that these isolates could be multiples of the same organism. By the other hand, although isolates C11, C12, C13.4, C19, C21.1, C22, C24.1, and C25 have been placed in the same cluster in the phylogenetic tree with zero distance, it is not sufficient to confirm that they are the same organism. Firstly because two distinct representative type strains (*A. punctata* and *A. caviae*) were placed together with these isolates, suggesting that 16S rRNA sequence alone could not provide enough taxonomic discriminatory power. Secondly, recent work has shown that multilocus phylogenetic analysis (MLPA) of at least five concatenated housekeeping genes is a more accurate tool for the delineation of *Aeromonas* species (Martinez-Murcia et al., 2011). Housekeeping genes evolve faster than the 16S rRNA, have a higher resolution for differentiating closely related species and therefore are more reliable for the correct identification of *Aeromonas* strains to species level. Finally, it can be seen from **Table 3** that only isolates C24.1 and C25 shared the same biochemical Vitek profile, while the closely related C11, C12, C13.4, C19, C21.1, and C22 isolates display some differences regarding presence of Ala–Phe–Pro–arylamidase, gama–glutamyl–transferase, tyrosine and glycine arylamidase, Glu–Gly–Arg– arylamidase, and L–lactate assimilation. In the case of the *Aeromonas* strains recovered in this study, the extent of clonal duplication in the isolate pools remains undetermined and additional studies using MLPA or genome sequencing will be necessary to identify which isolates are multiples of the same organism.

In this study, the bacterial isolates that displayed the greatest cellulolytic potential (**Table 2**) belong to the *Actinobacteria* phylum: R38A and R38E (*Microbacterium* species), I22A, I22B, I38C, I38D, I38E, and I37.1 (*Cellulosimicrobium* species), I1.2 (*Streptomyces* sp.); R7.1 (*Agromyces* sp.), and R38.2 (*Nocardiopsis* sp.). *Actinomycetes*, which are Gram positive filamentous bacteria, are well known for their ability to decompose complex molecules, particularly the lignocellulose components, which make them important agents in decomposition processes (Lacey, 1997). According to Pawar et al. (2012), very few (<1%) sequences in esophagus, crop, stomach, and rectum libraries were related to *Actinobacteria*, and in an intestine library they were completely absent. Cardoso et al. (2012a) showed that *Actinobacteria* were the minority phyla both inside the crop and the rectum (feces), but the intestinal bacterial community was not evaluated. Since the majority of our *Actinobacteria* isolates were obtained from the intestine lumen, this could explain the low percentage obtained in this previous work. Regardless, our results show that *Actinobacteria* representatives could be easily recovered from the intestinal tract of *A. fulica* and cultivated in order to produce a wide range of glycoside hydrolases. Although many members of the genus *Agromyces* have been isolated worldwide from soil (Li et al., 2003; Jurado et al., 2005; Yoon et al., 2008; Zhang et al., 2010), their cellulolytic capacities were not previously reported. Nevertheless, our enzymatic plate assay results showed that R7.1 degraded all the substrates tested, suggesting that *Agromyces* species can be valuable candidates for cellulase and xylanase production (**Table 2**). Interestingly, all of the *Aeromonas* isolates were capable of hydrolyzing CMC and the recalcitrant sugarcane bagasse, making them promising tools for the discovery of novel cellulases. This genus, unlike the relatively well characterized cellulolytic *Cellulosimicrobium* (Bakalidou et al., 2002; Kim do et al., 2012) and *Streptomyces* (Garda et al., 1997; Da Vinha et al., 2011), has been so far underestimated as a source of cellulolytic species. Our results show that the snail *A. fulica* is a source of distinct *Aeromonas* species that are very promising for cellulase production (**Tables 2**, **3** and **Figure 2**). Among 40 cellulolytic isolates retrieved in our study, a total of 10 isolates (I22A, I22B, I37.1, I38C, I38D, I38E, R7.1, R38.2, R38A, R38E) were able to hydrolyze all of the substrates tested in our plate assay, including the recalcitrant sugarcane bagasse, even without its pre-treatment (**Table 2**). This suggests that these isolates are able to secrete a bulk of lignocellulolytic enzymes that breaks the complex structure of the sugarcane cell wall. This enzymatic bulk may include endoglucanases,

cellobiohydrolases, and β-glucosidases for the hydrolysis of the cellulosic cell wall component; while other enzymes, such as endoxylanases and β-xylosidases could account for the hydrolysis of the hemicellulose fraction.

Based on a culture-dependent CMC-degrading bacteria screening method, this study for the first time demonstrates that the cellulolytic flora in the gastrointestinal tract of *A. fulica* can be easily recovered in order to produce several hydrolytic enzymes. Besides, this diversity changes according to the gut segment: while in the crop the proteobacteria *Aeromonas* was predominant, in the intestine the well characterized *Actinobacteria* phylum harbored the majority of the isolates, mainly *Cellulosimicrobium* genus representatives. This study extends the current knowledge of the *A. fulica* microbiota, and is the first investigation that specifically recovers cellulolytic bacteria from *A. fulica* by culture-dependent methods, making possible to use these isolates in fermentation processes for enzyme production or as source of novel genes for heterologous protein expression. Preliminary results of this study indicate that isolated bacteria are able to produce a diversity of enzymes and can degrade the highly recalcitrant sugarcane bagasse. Our future work will include the detailed genomic, biochemical and proteomic characterization of the secretome from selected isolates, in order to evaluate their lignocellulose-degrading potential on biotechnological processes.

Acknowledgments

We would like to thank the staff of Inmetro for technical assistance, insightful discussions and comments. This work was funded by the Brazilian research agencies Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ) and Conselho Nacional de Desenvolvimento Cientifico e Tecnologico (CNPq). We thank Joshua Nosanchuk for English revision.

Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.00860

References

Albuquerque, F. S., Peso-Aguiar, M. C., and Assunção-Albuquerque, M. J. (2008). Distribution, feeding behavior and control strategies of the exotic land snail *Achatina fulica* (*Gastropoda: Pulmonata*) in the northeast of Brazil. *Braz. J. Biol.* 68, 837–842. doi: 10.1590/s1519-69842008000400020

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990). Basic local alignment search tool. *J. Mol. Biol.* 215, 403–410. doi: 10.1016/S0022-2836(05)80360-2

Bakalidou, A., Kampfer, P., Berchtold, M., Kuhnigk, T., Wenzel, M., and Konig, H. (2002). *Cellulosimicrobium variabile* sp. nov., a cellulolytic bacterium from the hindgut of the termite *Mastotermes darwiniensis*. *Int. J. Syst. Evol. Microbiol.* 52, 1185–1192. doi: 10.1099/ijs.0.01904-0

Bruyne, C. K., and Loontiens, F. G. (1965). A fluorigenic substrate for β-D-xylosidase. *Naturwissenschaften* 52, 661–661. doi: 10.1007/BF00589630

Cann, I. K., Kocherginskaya, S., King, M. R., White, B. A., and Mackie, R. I. (1999). Molecular cloning, sequencing, and expression of a novel multidomain mannanase gene from *Thermoanaerobacterium polysaccharolyticum*. *J. Bacteriol.* 181, 1643–1651.

Cardoso, A. M., Cavalcante, J. J., Cantão, M. E., Thompson, C. E., Flatschart, R. B., Glogauer, A., et al. (2012b). Metagenomic analysis of the microbiota from the crop of an invasive snail reveals a rich reservoir of novel genes. *PLoS ONE* 7:e48505. doi: 10.1371/journal.pone.0048505

Cardoso, A. M., Cavalcante, J. J., Vieira, R. P., Lima, J. L., Grieco, M. A., Clementino, M. M., et al. (2012a). Gut bacterial communities in the giant land snail *Achatina fulica* and their modification by sugarcane-based diet. *PLoS ONE* 7:e33440. doi: 10.1371/journal.pone.0033440

Charrier, M., and Brune, A. (2003). The gut microenvironment of helicid snails (*Gastropoda: Pulmonata*): *in-situ* profiles of pH, oxygen, and hydrogen determined by microsensors. *Can. J. Zool.* 81, 08. doi: 10.1139/z03-071

Charrier, M., and Daguzan, J. (1980). Food consumption, production and energy evaluation in *Helix aspersa muller* (a terrestrial pulmonated gasteropod). *Ann. Nutr. Aliment.* 34, 147–166.

Charrier, M. Y., Fonty, G., Gaillard-Martinie, B., Ainouche, K., and Andant, G. (2006). Isolation and characterization of cultivable fermentative bacteria from the intestine of two edible snails, *Helix pomatia* and *Cornu aspersum* (Gastropoda: Pulmonata). *Biol. Res.* 39, 669–681. doi: S0716-97602006000500010

Chung, D. (1985). An anesthetic for internal operations on the land snail, *Helix aspersa müller*. *Veliger* 27, 5.

Da Vinha, F. N., Gravina-Oliveira, M. P., Franco, M. N., Macrae, A., da Silva Bon, E. P., Nascimento, R. P., et al. (2011). Cellulase production by *Streptomyces viridobrunneus* SCPE-09 using lignocellulosic biomass as inducer substrate. *Appl. Biochem. Biotechnol.* 164, 256–267. doi: 10.1007/s12010-010-9132-8

Deshpande, M. V., Eriksson, K. E., and Pettersson, L. G. (1984). An assay for selective determination of exo-1,4,-beta-glucanases in a mixture of cellulolytic enzymes. *Anal. Biochem.* 138, 481–487. doi: 10.1016/0003-2697(84)90843-1

Doi, R. H. (2008). Cellulases of mesophilic microorganisms: cellulosome and noncellulosome producers. *Ann. N.Y. Acad. Sci.* 1125, 267–279. doi: 10.1196/annals.1419.002

Edgar, R. C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* 32, 1792–1797. doi: 10.1093/nar/gkh340

Garda, A. L., Fernández-Abalos, J. M., Sanchez, P., Ruiz-Arribas, A., and Santamaria, R. I. (1997). Two genes encoding an endoglucanase and a cellulose-binding protein are clustered and co-regulated by a TTA codon in *Streptomyces halstedii* JM8. *Biochem. J.* 324(Pt 2), 403–411.

Gusakov, A. V., and Sinitsyn, A. P. (2012). Cellulases from *Penicillium* species for producing fuels from biomass. *Biofuels* 3, 463–477. doi: 10.4155/bfs.12.41

Hankin, L., and Anagnostakis, S. L. (1977). Solid media containing carboxymethylcellulose to detect CX cellulose activity of micro-organisms. *J. Gen. Microbiol.* 98, 109–115. doi: 10.1099/00221287-98-1-109

Heptinstall, J., Stewart, J. C., and Seras, M. (1986). Fluorimetric estimation of exo-cellobiohydrolase and β-d-glucosidase activities in cellulase from *Aspergillus fumigatus Fresenius*. *Enzyme Microb. Technol.* 8, 70–74. doi: 10.1016/0141-0229(86)90073-6

Holt, J. G. , Krieg, N. R., Sneath, P. H. A., Staley, J. T., and Williams, S. T. (eds.). (1994). *Bergey's Manual of Determinative Bacteriology. 9th Edn.* Baltimore, MD: Williams & Wilkins.

Hou, P., Li, Y., Wu, B., Yan, Z., Yan, B., and Gao, P. (2006). Cellulolytic complex exists in cellulolytic *Myxobacterium Sorangium*. *Enzyme Microb. Technol.* 38, 273–278. doi: 10.1016/j.enzmictec.2004.08.044

Jiang, Z., Dang, W., Yan, Q., Zhai, Q., Li, L., and Kusakabe, I. (2006). Subunit composition of a large xylanolytic complex (xylanosome) from *Streptomyces olivaceoviridis* E-86. *J. Biotechnol.* 126, 304–312. doi: 10.1016/j.jbiotec.2006.05.006

Jurado, V., Groth, I., Gonzalez, J. M., Laiz, L., and Saiz-Jimenez, C. (2005). *Agromyces subbeticus* sp. nov., isolated from a cave in southern Spain. *Int. J. Syst. Evol. Microbiol.* 55, 1897–1901. doi: 10.1099/ijs.0.63637-0

Kim do, Y., Ham, S. J., Kim, H. J., Kim, J., Lee, M. H., Cho, H. Y., et al. (2012). Novel modular endo-beta-1,4-xylanase with transglycosylation activity

from *Cellulosimicrobium* sp. strain HY-13 that is homologous to inverting GH family 6 enzymes. *Bioresour. Technol.* 107, 25–32. doi: 10.1016/j.biortech.2011.12.106

Ko, K. C., Han, Y., Choi, J. H., Kim, G. J., Lee, S. G., and Song, J. J. (2011). A novel bifunctional endo-/exo-type cellulase from an anaerobic ruminal bacterium. *Appl. Microbiol. Biotechnol.* 89, 1453–1462. doi: 10.1007/s00253-010-2949-9

Lacey, J. (1997). Actinomycetes in composts. *Ann. Agric. Environ. Med.* 4, 09.

Lancaster, C. R., and Simon, J. (2002). Succinate:quinone oxidoreductases from epsilon-proteobacteria. *Biochim. Biophys. Acta* 1553, 84–101. doi: 10.1016/S0005-2728(01)00230-4

Li, W. J., Zhang, L. P., Xu, P., Cui, X. L., Xu, L. H., Zhang, Z., et al. (2003). *Agromyces aurantiacus* sp. nov., isolated from a Chinese primeval forest. *Int. J. Syst. Evol. Microbiol.* 53, 303–307. doi: 10.1099/ijs.0.02350-0

Liang, Y. L., Zhang, Z., Wu, M., Wu, Y., and Feng, J. X. (2014). Isolation, screening, and identification of cellulolytic bacteria from natural reserves in the subtropical region of China and optimization of cellulase production by *Paenibacillus terrae* ME27-1. *Biomed. Res. Int.* 2014, 13. doi: 10.1155/2014/512497

Lucena, S. A., Lima, L. S., Cordeiro, L. S. Jr., Sant'anna, C., Constantino, R., Azambuja, P., et al. (2011). High throughput screening of hydrolytic enzymes from termites using a natural substrate derived from sugarcane bagasse. *Biotechnol. Biofuels* 4:51. doi: 10.1186/1754-6834-4-51

Luijten, M. L., de Weert, J., Smidt, H., Boschker, H. T., de Vos, W. M., Schraa, G., et al. (2003). Description of *Sulfurospirillum halorespirans* sp. nov., an anaerobic, tetrachloroethene-respiring bacterium, and transfer of Dehalospirillum multivorans to the genus Sulfurospirillum as Sulfurospirillum multivorans comb. nov. *Int. J. Syst. Evol. Microbiol.* 53, 787–793. doi: 10.1099/ijs.0.02417-0

Maki, M., Leung, K. T., and Qin, W. (2009). The prospects of cellulase-producing bacteria for the bioconversion of lignocellulosic biomass. *Int. J. Biol. Sci.* 5, 500–516. doi: 10.7150/ijbs.5.500

Martinez-Murcia, A. J., Monera, A., Saavedra, M. J., Oncina, R., Lopez-Alvarez, M., Lara, E., et al. (2011). Multilocus phylogenetic analysis of the genus *Aeromonas*. *Syst. Appl. Microbiol.* 34, 189–199. doi: 10.1016/j.syapm.2010.11.014

Pankratov, T. A., Tindall, B. J., Liesack, W., and Dedysh, S. N. (2007). *Mucilaginibacter paludis* gen. nov., sp. nov. and *Mucilaginibacter gracilis* sp. nov., pectin-, xylan- and laminarin-degrading members of the family Sphingobacteriaceae from acidic Sphagnum peat bog. *Int. J. Syst. Evol. Microbiol.* 57, 2349–2354. doi: 10.1099/ijs.0.65100-0

Pawar, K. D., Banskar, S., Rane, S. D., Charan, S. S., Kulkarni, G. J., Sawant, S. S., et al. (2012). Bacterial diversity in different regions of gastrointestinal tract of Giant African snail (*Achatina fulica*). *Microbiologyopen* 1, 415–426. doi: 10.1002/mbo3.38

Pérez-Avalos, O., Sánchez-Herrera, L. M., Salgado, L. M., and Ponce-Noyola, T. (2008). A bifunctional endoglucanase/endoxylanase from *Cellulomonas flavigena* with potential use in industrial processes at different pH. *Curr. Microbiol.* 57, 39–44. doi: 10.1007/s00284-008-9149-1

Rastogi, G., Muppid, G. L., Gurram, R. N., Adhikari, A., Bischoff, K. M., Hughes, S. R., et al. (2009). Isolation and characterization of cellulose-degrading bacteria from the deep subsurface of the Homestake gold mine, Lead, South Dakota, USA. *J. Ind. Microbiol. Biotechnol.* 36, 585–598. doi: 10.1007/s10295-009-0528-9

Robson, L. M., and Chambliss, G. H. (1989). Cellulases of bacterial origin. *Enzyme Microb. Technol.* 11, 626–644. doi: 10.1016/0141-0229(89)90001-X

Soares, F. L. Jr., Melo, I. S., Dias, A. C., and Andreote, F. D. (2012). Cellulolytic bacteria from soils in harsh environments. *World J. Microbiol. Biotechnol.* 28, 2195–2203. doi: 10.1007/s11274-012-1025-2

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* 28, 2731–2739. doi: 10.1093/molbev/msr121

Teather, R. M., and Wood, P. J. (1982). Use of Congo red-polysaccharide interactions in enumeration and characterization of cellulolytic bacteria from the bovine rumen. *Appl. Environ. Microbiol.* 43, 777–780.

Thiengo, S. C., Fernandez, M. A., Torres, E. J., Coelho, P. M., and Lanfredi, R. M. (2008). First record of a nematode *Metastrongyloidea* (*Aelurostrongylus abstrusus* larvae) in *Achatina* (*Lissachatina*) *fulica* (*Mollusca, Achatinidae*) in Brazil. *J. Invertebr. Pathol.* 98, 34–39. doi: 10.1016/j.jip.2007.10.010

Waeonukul, R., Kyu, K. L., Sakka, K., and Ratanakhanokchai, K. (2009). Isolation and characterization of a multienzyme complex (cellulosome) of the *Paenibacillus curdlanolyticus* B-6 grown on Avicel under aerobic conditions. *J. Biosci. Bioeng.* 107, 610–614. doi: 10.1016/j.jbiosc.2009.01.010

Weisburg, W. G., Barns, S. M., Pelletier, D. A., and Lane, D. J. (1991). 16S ribosomal DNA amplification for phylogenetic study. *J. Bacteriol.* 173, 697–703.

Yoon, J. H., Schumann, P., Kang, S. J., Park, S., and Oh, T. K. (2008). *Agromyces terreus* sp. nov., isolated from soil. *Int. J. Syst. Evol. Microbiol.* 58, 1308–1312. doi: 10.1099/ijs.0.65072-0

Zhang, D. C., Schumann, P., Liu, H. C., Xin, Y. H., Zhou, Y. G., Schinner, F., et al. (2010). *Agromyces bauzanensis* sp. nov., isolated from soil. *Int. J. Syst. Evol. Microbiol.* 60, 2341–2345. doi: 10.1099/ijs.0.017186-0

Zhang, M., Chekan, J. R., Dodd, D., Hong, P. Y., Radlinski, L., Revindran, V., et al. (2014). Xylan utilization in human gut commensal bacteria is orchestrated by unique modular organization of polysaccharide-degrading enzymes. *Proc. Natl. Acad. Sci. U.S.A.* 111, E3708–E3717. doi: 10.1073/pnas.1406156111

Zhang, Y. H. P., Himmel, M. E., and Mielenz, J. R. (2006). Outlook for cellulase improvement: screening and selection strategies. *Biotechnol. Adv.* 24, 452–481. doi: 10.1016/j.biotechadv.2006.03.003

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

APÊNDICE C - ARTIGO PUBLICADO NO JORNAL BRASILEIRO DE PATOLOGIA E MEDICINA LABORATORIAL (2015)

J Bras Patol Med Lab, v. 51, n. 3, p. 143-152, June 2015

10.5935/1676-2444.20150025

Methicillin- and vancomycin-resistant *Staphylococcus aureus* in health care workers and medical devices

Staphylococcus aureus *resistentes a meticilina (MRSA) e vancomicina (VRSA) em profissionais da saúde e artigos médicos*

Angela Breves; Catia Aparecida C. Miranda; Claudia Flores; Ivano de Filippis; Maysa M. Clementino

Instituto Nacional de Controle de Qualidade em Saúde (INCQS) – Fundação Oswaldo Cruz (Fiocruz), Rio de Janeiro, Brazil.

ABSTRACT

Introduction: Cross-contamination by *Staphylococcus aureus* among patients, professionals and medical supplies in health facilities is a constant concern, leading many researchers to study the prevalence of this pathogen in asymptomatic carriers. **Objectives:** We investigated the colonization and the antimicrobial susceptibility profile of *Staphylococcus* spp. on surfaces of medical articles and in professionals from two basic health units in the city of Rio de Janeiro. **Materials and methods:** Seventy-nine samples resulted in 49 isolates which underwent phenotypic and molecular characterization by polymerase chain reaction (PCR) of *coa*, *mecA* and *femA* genes. **Results:** According to the phenotypes, the isolates were identified as *S. aureus* (n = 35, 71.42%) and coagulase-negative *Staphylococcus* (CoNS) (n = 14, 28.57%). Among these 14 isolates, 42.85% were methicillin-resistant coagulase negative *Staphylococcus* (MRCoNS). Among the 35 *S. aureus*, 31.42% were methicillin resistant (MRSA), and 2.8% were vancomycin resistant, characterized as VRSA. Sixty-eight percent were susceptible to methicillin (MSSA). Genes *coa*, *femA* and *mecA* were amplified from 75.51%, 71.42% and 30.61% of the isolates, respectively. After amplification of the *mecA* gene, 20.41% were characterized as MRSA, and 10.20% as MRCoNS. The vancomycin-resistant strain was characterized as VRSA after detection of the *vanB* gene. **Conclusion:** Our results show a higher frequency of MSSA and MRCoNS among *S. aureus* and CoNS respectively, colonizing devices and health professionals. However, the already described transfer of the staphylococcal cassette chromosome *mec* (SCC*mec*) from MRCoNS to MSSA may alter these results, increasing the frequency of MRSA strains.

Key words: *Staphylococcus aureus*; MRSA; VRSA.

INTRODUCTION

Staphylococcus aureus is considered an opportunistic pathogen responsible for great morbidity and mortality; man is its main reservoir. It can be present in several sites of the human body, including oropharynx, intestines, hands, skin, and nasal cavity, which is pointed as one of the areas where colonization occurs more frequently[1, 2].

In health care settings, this pathogen may contaminate furniture, clothes and equipment around colonized or infected patients, which function as sources or reservoirs[3]. In this context, Murray *et al*. suggested that the health staff should use adequate techniques for hand washing, aimed at preventing *S. aureus* cross-infection among devices, professionals, and patients[4]. Gialluly *et al*. stressed the urgent need to alert and inform health care workers about the potential risk of hospital infection due to, mainly, lack of hand hygiene and handling of contaminated medical devices[5].

Since the study by Dr. Semmelweis, in the XIX century, health professionals' hands have been implicated as a source of microorganism transmission in health care settings[6]. In 1847, Semmelweis instituted hand-washing with chlorinated water as mandatory for all physicians, medicine students, and nurses, reducing mother mortality by puerperal fever from 12.2% to 2.4%, in the first month of intervention[7]. Since then, this procedure has been recommended as a primary measure to control dissemination of infectious agents.

Hand microbiota of mothers and health care workers at a maternity hospital revealed, among other organisms, the presence

First submission on 29/07/14; last submission on 09/03/15; accepted for publication on 09/04/15; published on 20/06/15

of *S. aureus* and coagulase-negative *Staphylococcus*, which have been pointed in the literature as associated with hospital infection outbreaks in nurseries[8].

Methicillin-resistant *Staphylococcus aureus* (MRSA) emerged as a nosocomial pathogen in the beginning of the 1960[9] decade, being first isolated in 1961[10]. In Brazil, MRSA isolates were detected in Barretos (São Paulo), in the hands of 73% of dentists and 52% of other dental professionals at basic health units in that city[11].

The main MRSA propagation mechanism in hospital settings operates by the hands of health care workers[12, 13]. There are reports in the literature about prevalence of MRSA colonization (4.6%) among physicians and nurses[14]. Braga *et al*., in 2004, highlighted the importance of hands as reservoir of microorganisms associated with cross-transmission of *S. aureus* by health care personnel[15].

MRSA strains are endemic in many American and European hospitals, representing around 30%-35% of all clinical isolates, in which infected or colonized patients are the main reservoir[16]. In Brazil there are scarce microbiological criteria for diagnosing hospital-acquired infections (HAIs)[17], but there is evidence that *S. aureus* is the most frequent agent and the most commonly transmitted by the hands of health professionals. However, few studies have been conducted to quantify this transmission[18]. Hand hygiene remains as the simplest and most important measure to prevent and reduce the risk of microorganism transmission between patients, and thus, the development of HAIs[19].

Dissemination of MRSA and oxacillin-resistant *Staphylococcus aureus* (ORSA) has been an object of studies in Brazil, aimed at verifying the frequency of resistance and implications in the hospital system due to the high percentage of HAIs caused by these microorganisms, what corresponds to 40%-80%, principally in intensive care units (ICUs)[20, 21].

Methicillin/oxacillin resistance in *S. aureus* and coagulase-negative *Staphylococcus* (CoNS) is firstly mediated by the production of penicillin-binding proteins (PBP2a), besides the normally produced proteins, PBP1, PBP2, PBP3 and PBP4, but with extremely low affinity for beta-lactam antibiotics, what hinders bacterial cell wall assembly[22]. The *mecA* gene, which encodes PBP2a, is highly conserved among methicillin-resistant *S. aureus* and *Staphylococcus epidermidis*, and is contained in a mobile genetic element, the staphylococcal cassette chromosome *mec* (SCC*mec*)[23]. There are some genes, called *factor essential for methicillin resistance* (*fem*), which help the *mecA* gene to express beta-lactam resistance. In 2011, a novel *mecA* homologue, termed *mecC*, located at type-XI SCC*mec*, was described in MRSA strains isolated in human beings and cattle[24]. Detection of the *mecC* gene in MRSA, as well as in other *Staphylococcus* species, has been performed in several countries[25].

The high prevalence of *S. aureus* strains and the consequent employment of vancomycin in Brazilian hospitals, added to the lack of control on antimicrobial use and the inadequate conditions of public health institutions, predispose to the emergence of strains of intermediate susceptibility (VISA), or resistant to vancomycin (VRSA)[26]. The reduced susceptibility to vancomycin in *S. aureus* (VISA) emerged in 1996, in Japan, where in the following year the first strain with heteroresistance to vancomycin (hVISA) was isolated, with a minimal inhibitory concentration (MIC) ≤ 2 µg/ml to vancomycin, but with subpopulations of MIC ≥ 4 µg/ml of approximately 10^{-5} to 10^{-6} cells. Because they are present at a much reduced number, they are not detected in the inocula used in the methodology recommended by the Clinical and Laboratory Standards Institute (CLSI)[27, 28].

In the subsequent years, isolated VISA strains were reported in the United States, France and Korea[29, 30]. In Brazil, the first report of multiple VRSA strains isolated at a hospital was the result of a study in 140 isolates from hospitalized patients exposed to vancomycin. Five of these isolates presented vancomycin MIC of 8 µg/ml, four of the VRSA strains were characterized as belonging to the Brazilian endemic clone, and all the five strains were negative for *vanA*, *vanB* and *vanC* genes by polymerase chain reaction (PCR)[31, 32].

In 2002, in the city of Michigan, United States, the first VRSA was isolated[33, 34]. This microorganism presented the *vanA* gene, suggesting the transfer of genetic material from *Enterococcus* spp., as the patient presented infection by vancomycin-resistant *Enterococcus* (VRE). In the latest years, many cases of VRSA have been reported, principally in hospital settings[27].

OBJECTIVES

This study aimed to investigate the prevalence and the susceptibility profile to antimicrobials in strains of negative-coagulase *S. aureus* and *Staphylococcus* sp. isolated from health professionals' hands and nostrils and from medical devices (stethoscope, sphygmomanometer, and Doppler machine) used at a pregnant women's health center in the city of Rio de Janeiro. In order to do so, we adopted phenotypic and molecular approaches to determine resistance profiles and classify phenotypes. From the results, we set out to point the risks associated with colonization by health professionals' resistant strains, and suggest corrective measures to prevent harm to the patients seen by these professionals.

MATERIALS AND METHODS

Study site and sample collection

The study was performed at two outpatient maternity clinics of two basic health centers in the region of Jacarepaguá, in the municipality of Rio de Janeiro. With the aid of dry sterile swabs, 79 samples were collected from the following medical appliances: stethoscope earpieces (n = 12, 24.49%), stethoscope diaphragm (n = 4, 8.16%), sphygmomanometer cuff (n = 4, 8.16%), sphygmomanometer bulb (n = 4, 8.16%), Doppler device (n = 9, 18.37%); and from health professionals: nostrils (n = 7, 14.29%) and hands (n = 9, 18.37%), from May 2009 to January 2010. After characterization, strains were stored at the collection of reference microorganisms on health surveillance (CMRVS) of Instituto Nacional de Controle de Qualidade em Saúde (INCQS) of Fundação Oswaldo Cruz (Fiocruz).

Approval by the research ethics committee

All health care workers that participated in the study filled and signed the free informed consent. The study was approved on October 15, 2009, by the research ethics committee (CEP) of the municipal health and civil defense office, under protocol nº 141/09, certificate of presentation for ethical consideration (CAAE): 0160.0.314.000-09. All the precepts contained in Resolution nº 196 of 1996 of the Ministry of Health[35], which guides researches with human beings, were respected.

Phenotypic characterization

Just after collection, the 79 samples were inoculated in test tubes containing brain-heart infusion (BHI) broth (Merck) and transported in a closed container at room temperature to the laboratory of reference microorganisms of INCQS/Fiocruz, as well as incubated at 37ºC for 24 hours. Later on, 0.1-ml aliquots were streaked onto mannitol salt agar (MSA) (Merck) and incubated at 37ºC for 24 hours. Among the cultures that fermented mannitol, two colonies were selected that underwent Gram stain, and catalase, deoxyribonuclease (DNase)[36] and free coagulase biochemical tests, with the addition of 0.5 ml of 24-hour-old culture in BHI broth to a tube containing 0.5 ml of rabbit plasma with ethylenediaminetetraacetic acid (EDTA) (Becton Dickinson), incubated at 37ºC in a thermostatic bath for 24 hours, taking readings each hour during five hours to verify clot formation. The absence of clot, in this period, led to the incubation of tubes for up to 24 hours. Reference *S. aureus* INCQS 00039 (ATCC 6538) (positive control) and *S. epidermidis* INCQS 00016 (ATCC 12228) (negative control) strains were used as controls.

Susceptibility to antimicrobials

Antimicrobial susceptibility tests were conducted by the modified Kirby-Bauer disk diffusion method (in agar), from a bacterial culture with a turbidity equivalent to a 0.5 McFarland standard in Mueller Hinton (MH) broth[37]. The reference strain *S. aureus* INCQS 00015 (ATCC 25923) was used as control according to CLSI[38]. The following antimicrobial agents were assessed: erythromycin (15 µg), clindamycin (2 µg), oxacillin (1 µg), vancomycin (30 µg), rifampicin (5 µg), chloramphenicol (30 µg), gentamicin (10 µg), ciprofloxacin (5 µg) and cefoxitin (30 µg) (Cefar, São Paulo-SP, Brasil). The results of vancomycin and teicoplanin susceptibility tests were interpreted in line with the table Sensifar and Multifar – Cefar®, according to the criteria recommended in the technical note of Agência Nacional de Vigilância Sanitária (Anvisa) nº 01/2010.

Vancomycin and teicoplanin MICs were determined by the Etest® (bioMérieux) system, using the reference strain *S. aureus* INCQS 000381 (ATCC 29213). After a culture was grown with microbial turbidity corresponding to a 0.5 McFarland standard in MH broth, the specimen was seeded with a swab over the surface of Petri plates containing MH agar, to which Etest® strips were applied, and incubated at 35ºC for 24 hours.

Molecular characterization

Genomic deoxyribonucleic acid (DNA) extraction and purification were performed from aliquots of 500 µl of each one of the 49 cultures, which were transferred to Eppendorf tubes, and centrifuged for 10 min at 5,000 g. The sediment was used for genomic DNA extraction with the DNeasy Blood & Tissue kit (Qiagen GmgH, Hildeitialln, Germany), according to the instructions by the manufacturer. Purified DNA samples were then stored at 20ºC for further use.

Detection of *coa*, *femA*, *mecA*, *vanA* and *vanB* genes

The PCR mixture had a final volume of 25 µl of the Master Mix M7505 kit (Promega) added with 20 pmol of each oligonucleotide, following instructions by the manufacturer. The used primers were synthesized by Invitrogen (Carlsbad, CA); amplifications were conducted in PTC-200 Peltier Thermal Cycler (MJ Research) and Eppendorf EP Master Cycler (**Table 1**). All PCR reactions were performed at least three times for assessment of reproducibility.

TABLE 1 – Genes, primers, and PCR conditions

Target gene	Primer	Sequence 5' ---- 3'	Program	Size (pb)	Reference
coa	*CoaG2* *CoaG3*	GAGACCAAGATTCAACAAG AAGAAAACCACTCACATCA	94ºC-2' 94ºC-30" 65ºC-2' 35× 72ºC-4' 72ºC-7'	900	Guler *et al*. (2005)[39]
femA	*FemAF* *FemAR*	TCACGCAACTGTTGGCCACT CCATTGCACTGCATAACTTCCGC	95ºC-5' 94ºC-2' 57ºC-2' 35× 72ºC-1' 72ºC-7'	700	This study
mecA	*mecAF* *mecAR*	GATCTGTACTGGGTTAATCA CATATGACGTCTATCCATTT3	95ºC-5' 94ºC-2' 57ºC-2' 30× 72ºC-1' 72ºC-7'	500	This study
vanA	*VanA1* *VanA2*	GGGAAAACGACAATTGC GTACAATGTGGCCGTTA	95ºC-5' 94ºC-1' 57ºC-2' 30× 72ºC-1' 72ºC-7'	732	Dutka-Malen *et al*. (1995)[40]
vanB	*VanB1* *VanB2*	ATGGGAAGCCGATAGTC GATTTCGTTCCTCGA CC	95ºC-2' 94ºC-1' 54ºC-1' 30× 72ºC-1' 72ºC-10'	635	Dutka-Malen *et al*. (1995)[40]

PCR: polymerase chain reaction.

The following strains were used for PCR control: *S. aureus* (MRSA) INCQS 00306 (ATCC 33591) and *S. epidermidis* INCQS 00016 (ATCC 12228). Electrophoresis was conducted in a horizontal Electrophoresis Cell (Bioamerica) apparatus containing 0.5× Tris/Borate/EDTA (TBE) buffer, for 60 minutes at 60 v with a Power Pac 300 (Bio-Rad). Images were digitized with the video documentation system ImageQuant 300®, GE.

Sequencing and identity analysis

The *vanB* gene PCR product was purified with a QIAquick® PCR Purification kit (Qiagen), according to the manufacturer's manual. The purified product underwent sequencing using the Big Dye Terminator kit for capillary electrophoresis in a ABI 3730 DNA Analyzer (Applied Biosystems, Foster City CA, USA) (Platform PDTIS/Fiocruz). The chromatogram was converted to Fasta format using the Sequencher 3.0 (Gene Codes Corporation, Ann Arbor, MI) software. Sequence similarity analysis was performed by BLASTn program (http://www.ncbi.nlm.nih.gov/BLAST/), at GenBank (National Center for Biotechnology Information [NCBI]).

RESULTS

Biochemical identification and antimicrobial susceptibility

The 79 samples collected from the two analyzed health units resulted in the isolation of 49 strains of *Staphylococcus* spp. (62%): 33 (67.34%) from medical devices, and 16 (32.66%) from health professionals. The 49 isolates presented morphology and staining characteristics of Gram-positive cocci, produced catalase and fermented mannitol. Forty-one isolates (83.67%) produced DNase enzyme; 35 (71.42%), coagulase enzyme; and the other 14 (28.57%) were negative for coagulase production. Regarding antimicrobial susceptibility, the 49 isolates presented 19 resistance profiles (**Table 2**). According to the presented phenotypes, the isolates were identified as *S. aureus* (n = 35, 71.42%) and CoNS (n = 14, 28.57%). Among these 14 isolates, six (42.85%) were methicillin-resistant coagulase-negative *Staphylococcus* (MRCoNS). Among the 35 *S. aureus*, 11 (31.42%) were MRSA; among these, one was also vancomycin resistant, and was identified as VRSA, and 24 (68.57%) were susceptible to methicillin (MSSA).

TABLE 2 – Antimicrobial resistance profiles of *Staphylococcus* spp. isolates

Profiles	Resistance phenotype	Nº of isolates
1	Sensitive to all	15
2	ERY	7
3	RIF	1
4	CHL	2
5	CLI	1
6	ERY and OXA	3[c]
7	ERY and GEN	1
8	ERY and RIF	1
9	OXA and CFO	1[c]
10	ERY, OXA and CFO	7[b]
11	ERY, CHL and CLI	1
12	ERY, GEN and CFO	2
13	ERY, OXA, GEN and CFO	1[a]
14	ERY, CHL, CLI and RIF	1
15	ERY, CLI, OXA, RIF and CFO	1[a]
16	ERY, CLI, OXA, CIP and CFO	1[c]
17	ERI, CLO, CLI, OXA, RIF and CFO	1[a]
18	ERI, CLI, OXA, VAN, RIF, CFO and TEI	1[a]
19	ERI, CLI, OXA, GEN, CIP, RIF and CFO	1[a]
Total		49

[a]All MRSA strains; [b]five MRSA strains and one MRCoN; [c]all MRCoN strains; ERY: erythromycin; RIF: rifampicin; CHL: chloramphenicol; CLI: clindamycin; GEN: gentamicin; OXA: oxacillin; CFO: cefoxitin; CIP: ciprofloxacin; VAN: vancomycin; TEI: teicoplanin; MRSA: methicillin-resistant Staphylococcus aureus*; MRCoN: methicillin-resistant coagulase-negative* Staphylococcus.

The strains that presented resistance to five or more antibiotic classes were considered multiresistant. One single isolate, from the hand of a physician, presented resistance to vancomycin, with CIM ≥ 256 µg/ml, and to five other analyzed antibiotics: oxacillin, erythromycin, clindamycin, rifampicin and cefoxitin; resistance to teicoplanin was also verified, with CIM ≥ 256 µg/ml. This isolate was stored at CMRVS, under access number P3425.

Identification of *femA*, *coa*, *mecA*, *vanA* and *vanB* genes

Among the 49 isolates, 35 (71.4%) presented a single 900-bp fragment compatible with *femA* gene. PCR of the *coa* gene resulted in the amplification of a single fragment of 650-900 bp in 37 (75.51%) of the analyzed strains. A 500-bp fragment corresponding to the *mecA* gene was detected in 15 (30.6%) strains; among these, one isolate (6.66%) revealed the presence of a 635-bp fragment corresponding to the *vanB* gene. The presence of a *vanA* gene specific fragment was not verified. The sequence of the *vanB* gene presented 98% identity with sequences of this gene at GeneBank, where it was stored under KP 731622 access number.

Characterization of *Staphylococcus* spp.

After phenotypical and molecular identification, the 49 isolates were identified as MSSA ($n = 27$), MRSA ($n = 10$) – one of these isolates was also identified as VRSA –, CoNS ($n = 7$) and MRCoNS ($n = 5$). Among these isolates, 35 produced coagulase (*S. aureus*) and 14 did not (CoNS), by the conventional method of free coagulase. However, the *coa* gene was amplified in 37 isolates, which were then identified as *S. aureus*. Among the 49 strains, 15 presented the *mecA* gene. Thirty-seven isolates were characterized as *S. aureus*; among them, 10 presented the *mecA* gene, and were identified as MRSA; another five were considered MRCoNS.

DISCUSSION

Staphylococcus aureus is part of the skin microbiota of up to a third of the general population; the nasal vestibules (35%) and the perianal region (30%) are the main reservoirs, followed by the axillary and interdigital regions (5%-10%), where dissemination can occur, causing infections[41]. Therefore, infections in healthcare settings caused by multiresistant *S. aureus* have become quite relevant in the latest decades, being responsible for high indices of morbidity and mortality[42-44].

In health centers, the main reservoirs of *S. aureus* are the infected patients, although physicians, nurses and other staff members may be reservoirs and elements of propagation and maintenance. In this perspective, it is worth emphasizing that prevention of *S. aureus* infection depends principally on the mechanisms for controlling environment and healthy carriers[76]. Oxacillin or methicillin-resistant (MRSA) isolates are among the major pathogens causing infections in the world, leading to the emergence of and disseminating increasingly virulent and multiresistant strains[46].

In this study, the use of a polyphasic identification approach allowed the detection of *Staphylococcus* spp., presenting MRSA, VRSA, MSSA, CoNS, and MRCoNS phenotypes, in isolates from both equipment and health professionals. For example, presence of coagulase (71.42%) and detection of the *coa* gene (75.51%) made us suggest that although the coagulase test in tubes is considered the standard method for differentiation of *Staphylococcus* spp., it

may present false negative results, as demonstrated in our study, in which coagulase-negative strains revealed the presence of the *coa* gene by PCR[80].

Detection of the *coa* gene has been adopted in species differentiation and typing because it is considered accurate and often more sensitive than detection of coagulase by biochemical assays[48, 49]. Besides being a factor of virulence, the gene encoding coagulase synthesis is present in several allelic forms, what makes it possible for isolates to be classified into different variants. Like the *spa* (protein A) gene, the *coa* gene has a polymorphic region used for differentiation of *S. aureus* isolates with the analysis of polymorphism of length of restriction fragments, a method used in epidemiologic studies[49-51].

In this study, the presence of the *femA* gene in 35 *S. aureus* isolates was confirmed. It is present exclusively in this species and is used for the selective detection of this microorganism[52-54], although homologues of this gene have been characterized into CoNS species, such as *S. epidermidis*, *S. simulans*, *S. hominis* and *S. saprophyticus*[55]. The absence of the *femA* gene from two coagulase-negative isolates that presented the *coa* gene may be due to variations in the annealing regions of primers, making the amplification of *femA* impossible[56].

Our results demonstrated that among the 17 oxacillin-resistant (ORSA) isolates, 11 (64.7%) expressed coagulase; and 10 (58.8%), the *mecA* gene, being identified as MRSA. The oxacillin-resistant strain, which did not present the *mecA* gene, leads us to suggest the presence of another mechanism of oxacillin resistance, as, for instance, hyperproduction of beta-lactamase, modification of the PBP binding site or presence of the *mecC* gene[24, 57-60].

In Brazil, MRSA dissemination has been object of studies aimed at verifying the frequency of resistance and its implications in the health system[20, 21, 41, 61]. Two studies in Rio Grande do Sul demonstrated the presence of 32.7% MRSA in hospitalized patients, and 20.6% in saliva of 13 cleaning workers at the hospital[61, 62]. In Pernambuco, the prevalence rate was 13% in ICU inpatients, while in Bahia, this rate was 28%[2, 20]. An investigation of nasal swabs of hospitalized newborn at a maternity hospital in Rio de Janeiro verified a frequency of 47% of this pathogen[63]. The presence of MRSA was also detected in hands and buccal cavity of 73% of dentists, 52% of other health professionals and 54% of patients at dental clinics of basic health centers in the city[11].

In recent decades, vancomycin has been the drug of choice for the treatment of MRSA infections. However, the excessive use of this antibiotic has led to increased resistance in *Enterococcus*, CoNS and MRSA strains. VISA isolates were initially detected in Japan, United States, France, South Africa and South Korea, from single patients or groups of patients in a same hospital, demonstrating the transfer capacity of this organism and drawing attention to the importance of improving control measures for nosocomial infections[64-67].

In Brazil, the presence of vancomycin tolerance in ORSA strains was determined in 49.1% of the 395 hospital isolates in São Paulo, what certainly increases the risk of failures in treatments with vancomycin, besides increasing the risk of emergence of VISA[31]. In Brazil, HAIs by these microorganisms were initially reported in São Paulo and Rio de Janeiro. Later, an outbreak of VISA was described at a hospital in São Paulo, with isolation of four strains at a burn center[31, 68, 69]. The first report of multiple VRSA isolates at a Brazilian hospital was the result of a study in 140 isolates of inpatients exposed to vancomycin[32]. At the present study, one of the most surprising pieces of data was the detection of an isolate – VRSA – colonizing the hand of a physician at the health care center. This asymptomatic colonization is highly clinically significant, as the individual colonized in the nostrils may contaminate his own hands and become a vehicle for this pathogen via the mechanism of contact infection. Consequently, the pathogen dissemination can occur in the health care setting, where there is circulation of individuals and patients who are more susceptible to exogenous infection[32].

A study involving a thousand healthy individual also demonstrated 22.5% and 16.6% of individuals colonized by *Staphylococcus aureus* and MRSA, respectively, in nostrils, forearm and hands[70]. Concern over VRSA colonization and transmission is not caused exclusively by nasal carriers, but also by medical devices as vehicles of this transmission. Thus, this colonization is considered a public health problem, and it is of interest to investigate whether health professionals are also nasal carriers of MRSA[71].

Studies about the epidemiological role of hands in the transmission of infection among health care workers have recognized their potential as source of eventual hospital infections, as well as the possible relationship between isolates from different anatomical locations of the same individual, mainly between nasal cavities and hand[1]. Besides, hands are also considered one of the main sources of cross-transmission of nosocomial infections among patients, equipment and/or contaminated surfaces, attributed to inadequate hygiene processes[72-74].

Awareness of the risks of infection transmission, limitations of disinfection methods, and difficulties of processing inherent in the nature of each medical device is fundamental for the adequate measures to be taken[75]. Although hand hygiene has been the most important and recognized measure for prevention and control of infections, mainly by *S. aureus*, strong resistance to it is still observed before or after handling of patients in health services. Therefore, putting hand hygiene in practice has been a complex and

difficult task, as professionals need to be aware of its importance in health care settings for safety and quality of care[76, 77].

The main prevention measures to control the spread of multiresistant microorganisms include: laboratory data-based surveillance, isolation of infected or colonized patients, use of barrier precautions (gloves and caps), hand washing and antisepsis, and cleaning the environment near the patient[78]. Inherent in all control measures of HAIs, education of health care workers is very important to the correct performance of patient care duties[79]. The factors associated with poor adherence to hand hygiene are, principally, heavy workloads, glove use, and conduction of activities involving cross transmission during specialized techniques[80].

The implementation of control and prevention measures must include continuous education, monitoring of adherence to hand hygiene practices, besides feedback of data, installation and maintenance of equipment, rational use of antibiotics, and recommendations based on caution in invasive procedures[81].

CONCLUSION

Our results showed higher frequency of MSSA and MRCoNS among *S. aureus* and CoNS isolates, respectively, colonizing equipment and health care workers. However, the already described SSC*mec* transfer from MRCoNS to MSSA[82] could alter these results, increasing the frequency of methicillin-resistant strains.

As far as we know, this is the first report about isolation of a VRSA strain presenting the vancomycin resistance gene *vanB*, with CIM ≥ 256 µg/ml to vancomycin and teicoplanin, from the hands of a health care worker in Brazil.

As a result, we demonstrate the increased dissemination of resistance, and conclude that the adoption of procedures for hand antisepsis and disinfection of medical items is essential to prevent dissemination of these pathogens among patients, health professionals, and individuals of the community.

ACKNOWLEDGEMENTS

We thank Secretaria Municipal de Saúde e Defesa Civil of the state of Rio de Janeiro, the direction and health professionals of the municipal health centers that participated in this study. We also thank the financial support of Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (474204/2012-0), Fundação Carlos Chagas de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ) (E-26/112.657/2012), and INCQS-Fiocruz.

RESUMO

Introdução: *A contaminação cruzada por* Staphylococcus aureus *entre pacientes, profissionais e materiais de uso médico em unidades de saúde é uma preocupação constante, o que leva pesquisadores a estudar a prevalência desse patógeno em portadores assintomáticos.* ***Objetivos:*** *Investigamos a colonização e o perfil de suscetibilidade aos antimicrobianos de* Staphylococcus *spp. em superfícies de artigos médicos e em profissionais de duas unidades básicas de saúde no município do Rio de Janeiro.* ***Materials e métodos:*** *Foram coletadas 79 amostras que resultaram em 49 isolados, submetidos à caracterização fenotípica e molecular por meio da reação em cadeia da polimerase (PCR) dos genes* coa*,* femA *e* mecA*.* ***Resultados:*** *De acordo com os fenótipos apresentados, os isolados foram identificados como* S. aureus *(*n *= 35; 71,42%) e* Staphylococcus *coagulase negativa (CoNS) (*n *= 14; 28,57%). Destes 14 isolados, 42,85% foram* Staphylococcus *coagulase negativa resistentes a meticilina (MRCoNS). Dos 35* S. aureus*, 31,42% foram resistentes a meticilina (MRSA). Uma cepa foi resistente a vancomicina e identificada como* S. aureus *resistente a vancomicina (VRSA) após a detecção do gene* vanB*. Sessenta e oito por cento foram suscetíveis a meticilina (MSSA). Os genes* coa*,* femA *e* mecA *foram amplificados em 75,51%; 71,42% e 30,61% dos isolados, respectivamente. Após amplificação do gene* mecA*, 20,41% foram classificados como MRSA e 10,20% como MRCoNS.* ***Conclusão:*** *Nossos resultados mostraram frequência maior de MSSA e MRCoNS entre* S. aureus *e CoNS, respectivamente, colonizando equipamentos e profissionais de saúde. No entanto, a já descrita transferência do cassete cromossômico estafilocócico* mec *(SSC*mec*) de MRCoNS para MSSA poderia alterar esses resultados, aumentando a frequência de cepas MRSA.*

Unitermos: Staphylococcus aureus*; MRSA; VRSA.*

REFERENCES

1. Santos BMO. Monitoramento da colonização pelo Staphylococcus aureus em alunos de um curso de auxiliar de enfermagem durante a formação profissional. Rev Latino-Am Enfermagem 2000; 8(1): 67-73.

2. Cavalcanti SMM, França ER, Vilela MA, Montenegro F, Cabral C, Medeiros, ACR. Estudo comparativo da prevalência de Staphylococcus aureus importado para as unidades de terapia intensiva de Hospital Universitário, Pernambuco, Brasil. Rev Bras Epidemiol 2006; 9: 436-46.

3. Gomes MZ, Oliveira LA. Infecção estafilocócica. In: Lopes AC, editor. Tratado de clínica médica. v. 3. São Paulo: Roca Ltda; 1996. p. 3974-95.

4. Murray PR, Rosenthal KS, Kobayashi GS, Pfaller MA. Microbiologia médica. 3 ed. Rio de Janeiro: Guanabara Koogan; 2000, 151 p.

5. Gialluly C, Morange V, Gialluly E, Loulergue J, van der Mee N, Quentin R. Blood pressure cuff as a potential vector of pathogenic microorganisms: a prospective study in a teaching hospital. Infect Control Hosp Epidemiol 2006; 27: 940-3.

6. Elias J, Claus H, Frosch M, Vogel U. Evidence for indirect nosocomial transmission of Neisseria meningitidis resulting in two cases of invasive meningococcal disease. J Clin Microbiol. 2006; 44: 4276-8.

7. Andrade D, Angerami ELS. Reflexões acerca das infecções hospitalares às portas do terceiro milênio. Rev Med, Ribeirão Preto. 1999; 32: 492-7.

8. Palos MAP. Microbiota das mãos de mães e de profissionais de saúde de uma maternidade de Goiânia. Rev Eletr Enf. 2009; 11(3): 573-8.

9. Lane WR. Methicillin resistance in staphylococci. Med J Aust. 1962; 49(1): 962-5.

10. Jevons MP. Celbenin resistant staphylococci. BMJ. 1961; 1(5219): 124-5.

11. Lancellotti M. Estudo epidemiológico de Staphylococcus spp. em ambientes, água e portadores sadios e determinação da sensibilidade a antimicrobianos [tese]. Jaboticabal: Faculdade de Ciências Agrárias e Veterinárias, Universidade Estadual Paulista; 2006.

12. Becker K, Friedrich AW, Lubritz G, Weilert M, Peters G, Von Eiff C. Prevalence of genes encoding pyrogenic toxin superantigens and exfoliative toxins among strains of Staphylococcus aureus isolated from blood and nasal specimens. J Clin Microbiol. 2003; 41(4): 1434-9.

13. Benício CG, Reis C, Pimenta FC. Isolamento e contagem de Staphylococcus sp. na saliva de acadêmicos de Medicina. Braz J Infect Dis. 2003; 7(S1): S91.

14. Albrich WC, Harbarth S. Health-care workers: source, vector, or victim of MRSA? Lancet Infect Dis. 2008; 8(5): 289-301.

15. Braga KAM, Souza LBS, Santana WJ, et al. Microrganismos mais frequentes em Unidades de Terapia Intensiva. Rev Med Ana Costa. 2004; 9: 71-4.

16. Oztoprak N, Cevik MA, Akinci E, et al. Risk factors for ICU-acquired methicillin-resistant Staphylococcus aureus infections. Am J Infec Control. 2006; 34(1): 1-5.

17. Gontijo Filho PP. Problemas de vigilância epidemiológica de infecção hospitalares sem o uso de critérios microbiológicos no Brasil. Rev Cien Farm Basil Aplic. 2006; 27: 97-102.

18. Gleeson TD. Prevention and control of methicillin-resistant Staphylococcus aureus. Dis Mon. 2008; 54(12): 801-6.

19. Silvestri L, Petros AJ, Sarginson RE, de la Cal MA, Murray AE, van Saene HK. Hand washing in the intensive care unit: a big measure with modest effects. J Hosp Infect. 2005; 59(3): 172-9.

20. Brites C, Silva N, Sampaio-Sá M. Temporal evolution of the prevalence of methicillin-resistant Staphylococcus aureus in a tertiary hospital in Bahia, Brazil: a nine-year evaluation study. Braz J Infect Dis. 2006; 10: 235-8.

21. Oliveira DC, Milheirico C, De Lencastre H. Redefining a structural variant of staphylococcal cassette chromosome mec, SCCmec type VI. Antimicrob Agents Chemother. 2006; 50: 3457-9.

22. Hackbarth CJ, Chambers HF. Methicillin-resistant staphylococci: genetics and mechanisms of resistance. Antimicrob Agents Chemother. 1989; 33: 991-4.

23. Ryffel C, Tesch W, Birch-Machin I, et al. Sequence comparison of mecA genes isolated from methicillin-resistant Staphylococcus aureus and Staphylococcus epidermidis. Gene. 1990; 94(1): 137-8.

24. García-Álvarez L, Holden MT, Lindsay H, et al. Meticillin-resistant Staphylococcus aureus with a novel mecA homologue in human and bovine populations in the UK and Denmark: a descriptive study. Lancet Infect Dis. 2011; 11(8): 595-603.

25. Vanderhaeghen W, Vandendriessche S, Crombé F, et al. Characterization of methicillin-resistant non-Staphylococcus aureus staphylococci carriage isolates from different bovine populations. J Antimicrob Chemother. 2013; 68(2): 300-7.

26. Oliveira GA, Levy CE, Mamizuka EM. Staphylococcus aureus apresentando resistência intermediária à vancomicina: mecanismos de resistência, detecção laboratorial e perspectivas de emergência no Brasil. J Bras Patol. 2000; 36(2): 96-102.

27. Tenover FC, Moellering RC Jr. The rationale for revising the Clinical and Laboratory Standards Institute vancomycin minimal inhibitory concentration interpretive criteria for Staphylococcus aureus. Clin Infect Dis. 2007; 44(9): 1208-15.

28. Howden BP, McEvoy CR, Allen DL, et al. Evolution of multidrug resistance during Staphylococcus aureus infection involves mutation of the essential two component regulator WalKR. PLoS Pathog. 2011; 7(11).

29. Chang FY, Peacock JE Jr, Musher DM. Staphylococcus aureus bacteremia: recurrence and the impact of antibiotic treatment in a prospective multicenter study. Medicine. 2003; 82: 333-9.

30. Sakoulas G, Moellering RC Jr. Increasing antibiotic resistance among methicillin-resistant Staphylococcus aureus strains. Clin Infect Dis. 2008; 46: S360-7.

31. Oliveira GA, Okada SS, Guenta RS, Mamizuka EM. Avaliação da tolerância à vancomicina em 365 cepas hospitalares de Staphylococcus aureus resistentes à oxacilina. J Bras Patol Med Lab. 2001; 37(4): 239-46.

32. Oliveira GA, Dell'Aquila AM, Masiero RL, et al. Isolation in Brazil of nosocomial Staphylococcus aureus reduced susceptibility to vancomycin. Infect Control Hosp Epidemiol. 2001a; 22(7): 443-8.

33. Mimica MJ, Mendes CMF. Diagnóstico laboratorial da resistência à oxacilina em Staphylococcus aureus. J Bras Patol Med Lab. 2007; 43(6): 399-406.

34. Santos AL, Santos DO, Freitas CC, et al. Staphylococcus aureus: visitando uma cepa de importância hospitalar. J Bras Patol Med Lab. 2007; 43(6): 413-23.

35. Ministério da Saúde (BR). Resolução do Conselho Nacional de Saúde nº 196, de 10 de outubro de 1996. Aprova as diretrizes e normas regulamentadoras de pesquisas envolvendo seres humanos. Brasília (DF): Diário Oficial [da] República Federativa do Brasil; 1996, Seção 1, p. 21082-5.

36. Koneman EW, Allen SD, Janda WM, Schreckenberger PC, Win WC. Diagnóstico microbiológico: texto e atlas colorido. 6 ed. Rio de Janeiro: Medsi; 2008, p. 551-88.

37. Bauer AW, Kirby WM, Sherris JC, Turck M. Antibiotic susceptibility testing by a standardized single disk method. Am J Clin Pathol. 1966; 45(4): 493-6.

38. Clinical and Laboratory Standards Institute. Performance standards for antimicrobial disk susceptibility testing; approved standard. 30 ed. Document M100-S20. Wayne (PA): Clinical and Laboratory Standards Institute.

39. Güler L, Ok U, Gündüz K, Gülcü Y, Hadimli HH. Antimicrobial susceptibility and coagulase gene typing of Staphylococcus aureus isolated from bovine clinical mastitis cases in Turkey. J Dairy Scien. 2005; 88: 3149-54.

40. Dutka-Malen S, Evers S, Courvalin SP. Detection of glycopeptide resistance genotypes and identification to the species level of clinically relevant enterococci by PCR. J Clin Microbiol. 1995; 33: 24-7.

41. Cavalcanti SM, França ER, Cabral C, et al. Prevalence of Staphylococcus aureus introduced into intensive care units of a university hospital. Braz J Infect Dis. 2005; 9: 1: 56-63.

42. Grundmann H. Geographic distribution of Staphylococcus aureus causing invasive infections in Europe: a molecular-epidemiological analysis. PloS Med. 2010; 7: 1-15.

43. Michelin DC, Moreschi PE, Lima AC, Nascimento GGF, Paganelli MO, Chaud MV. Avaliação da atividade antimicrobiana de extratos vegetais. Rev Bras Farmac. 2005; 15: 316-20.

44. Martins A, Cunha MLRS. Methicillin resistance in Staphylococcus aureus and coagulase-negative Staphylococci: epidemiological e molecular aspects. Microbiol Immunol. 2007; 51(9): 787-95.

45. Tanaka M, Hayashida Y, Iguchi T, Nakao N, Nakai N, Nakashima K. Organization of the mouse ghrelin gene and promoter: occurrence of a short noncoding first exon. Endocrinology. 2001; 142(8): 3697-700.

46. Velázquez-Meza ME. Staphylococcus aureus methicillin-resistant: emergence and dissemination. Salud Pública Méx. 2005; 47(5): 381-7.

47. Vannuffel P, Gigi J, Ezzedine H, et al. Specific detection of methicillin-resistant Staphylococcus species by multiplex PCR. J Clin Microbiol. 1995; 33: 2864-7.

48. Vieira-da-Motta O, Folly MM, Sakyiama CCH. Detection of different Staphylococcus aureus strains in bovine milk from subclinical mastitis using PCR and routine techniques. Bras J Microbiol. 2001; 32(1): 27-31.

49. Gharib AA, Adel Attia MA, Bendary MM. Detection of the coa gene in Staphylococcus aureus from different sources by polymerase chain reaction. International J Microbiol Res. 2013; 4: 37-42.

50. Goh SH, Byrne SK, Zhang JL, Chow AW. Molecular typing of Staphylococcus aureus on the basis of coagulase gene polymorphism. J Clin Microbiol. 1992; 30: 1642-5.

51. Hookey JV, Richardson JF, Cookson BD. Molecular typing of Staphylococcus aureus based on PCR restriction fragment length polymorphism and DNA sequences analysis of the coagulase gene. J Clin Microbiol. 1998; 36: 1083-9.

52. Browne DFJ, Brown L. Evaluation of E-test, a novel method for quantifying antimicrobial activity. J Antimicrob Chemother. 1991; 27: 185-90.

53. Leclercq R, Derlot E, Duval J, Courvalin P. Plasmid-mediated resistance to vancomycin and teicoplanin in Enterococcus faecium. N Engl J Med. 1988; 319: 157-61.

54. Mulligan ME. Methicillin-resistant Staphylococcus aureus: a consensus review of the microbiology, pathogenesis, and epidemiology with, implications for prevention and management. Am J Med. 1993; 94(3): 313-28.

55. Alborn WE Jr, Hoskins J, Unal S. Cloning and characterization of femA and femB from Staphylococcus epidermidis. Gene. 1996; 180(1-2): 177-81.

56. Vanuffel P, Heusterspreute M, Bouyer M, Vandercam B, Philippe M, Gala JL. Molecular characterization of femA from Staphylococcus hominis and Staphylococcus saprophyticus, and femA-based discrimination of staphylococcal species. Res Microbiol. 1999; 150(2): 129-41.

57. Geha DJ. Multiplex PCR for identification of methicillin-resistant staphylococci in the clinical laboratory. J Clin Microbiol. 1994; 32: 1768-72.

58. Jorgensen JH. Selection criteria for an antimicrobial susceptibility testing system. J Clin Microbiol. 1993; 31: 2841-4.

59. Mohanasoundaram KM, Lalitha MK. Comparison of phenotypic versus genotypic methods in the detection of methicillin resistance in Staphylococcus aureus. Indian J Med Res. 2008; 27: 78-84.

60. Shore AC, Deasy EC, Slickers P. Detection of staphylococcal cassette chromosome mec type XI carrying highly divergent mecA, mecI, mecR1, blaZ, and ccr genes in human clinical isolates of clonal complex 130 methicillin-resistant Staphylococcus aureus. Antimicrob Agents Chemother. 2011; 55(8): 3765-73.

61. Spiandorello WP, Morsh F, Sebben S, Spiandorello FSA. A resistência do Staphylococcus aureus à oxacilina em hospital de Caxias do Sul. Rev AMRIGS. 2000; 44(3-4): 120-5.

62. Cruz EDA, Pimenta FC, Hayashida M, Eidt M, Gir E. Detecção de Staphylococcus aureus na boca de trabalhadores da limpeza hospitalar. Rev Lat Am Enferm. 2011; 19: 1-7.

63. Loureiro MM, Moraes BA, Quadra MRR, Pinheiro GS, Suffys PN, Asensi MD. Molecular epidemiology of methicillin resistant Staphylococcus aureus isolated from newborns in a hospital in Rio de Janeiro, Brazil. Mem Inst Oswaldo Cruz. 2000; 95(6): 777-82.

64. Hiramatsu K, Aritaka N, Hanaki H, et al. Dissemination in Japanese hospitals of strains of Staphylococcus aureus heterogeneously resistant to vancomycin. Lancet. 1997; 350: 1670-3.

65. Ploy MC, Grélaud C, Martin C, de Lumley L, Denis F. First clinical isolate of vancomycin-intermediate Staphylococcus aureus in a French hospital. Lancet. 1998; 351: 1212.

66. Tenover FC, Lancaster MV, Hill BC, et al. Characterization of staphylococci with reduced susceptibilities to vancomycin and other glycopeptides. J Clin Microbiol. 1998; 36: 1020-7.

67. Rotun SS, McMath V, Schoonmaker DJ, et al. Staphylococcus aureus with reduced susceptibility to vancomycin isolated from a patient with fatal bacteremia. Emerg Infect Dis. 1999; 5: 147-9.

68. Del'Alamo L, Cereda RF, Tosin I, Miranda EA, Sader HS. Antimicrobial susceptibility of coagulase-negative staphylococci and characterization of isolates with reduced susceptibility to glycopeptides. Diagn Microbiol Infect Dis. 1999; 34: 185-91.

69. Nunes APF. S. aureus coagulase-negativos: diferenciação em espécies, diversidade genômica de espécies resistentes à oxacilina e determinação da sensibilidade à glicopeptídios [dissertação]. Rio de Janeiro: Instituto de Microbiologia Prof. Paulo de Góes, Universidade Federal do Rio de Janeiro; 2000.

70. Gould IM, Cauda R, Esposito S, Gudiol F, Mazzei T, Garau J. Management of serious meticillin-resistant Staphylococcus aureus infections: what are the limits? Int J Antimicrob Agents. 2011; 37(3): 202-9.

71. Neves MC, Rossi Júnior OD, Alves ECC, Lemos MVF. Detecção de genes de resistência antimicrobiana em cromossomos e plasmídeos de Staphylococcus spp. Arq Inst Biol. 2007; 74(3): 207-13.

72. Bjerke BN. The evolution: handwashing to hand hygiene guidance. Crit Care Nurs Q. 2004; 27: 295-307.

73. Karabey S, Ay P, Derbentli S, Nakipoglu Y, Esen F. Handwashing frequencies in an intensive care unit. J Hosp Infect. 2002; 50: 36-41.

74. Larson EL. Hygiene of skin: when is clean too clean. Emerg Infect Dis. 2001; 7(2): 225-30.

75. Lacerda RA. Brazilian scientific production on nosocomial infection and nursing contribution: past, present and perspectives. Rev Lat Amer Enferm. 2002; 10: 55-63.

76. Agência Nacional de Vigilância Sanitária. Resolução da Diretoria Colegiada da Agência Nacional de Vigilância Sanitária nº 14, de 28 de fevereiro de 2007. Aprova o regulamento técnico para produtos saneantes com ação antimicrobiana harmonizado no âmbito do MERCOSUL através da resolução GMC nº 50/06. Brasília (DF): Diário Oficial [da] República Federativa do Brasil; 2007, Seção 1.

77. Maskerine C, Loeb M. Improving adherence to hand hygiene among health care workers. J Contin Educ Health Prof. 2006; 26(3): 244-51.

78. Boyce JM. MRSA patients: proven methods to treat colonization and infection. J Hosp Infect. 2001; 48: 9-14.

79. Mayhall CG. Hospital epidemiology and infection control. 3 ed. Philadelphia: Lippicott-Raven; 2004, 2060 p.

80. Rangle J, Clarke M, Storr J. Hand hygiene compliance in healthcare workers. J Hosp Infect. 2006; 64: 205-9.

81. Pittet D. Infection control and quality health care in the new millennium. Am J Infec Control. 2005; 33(5): 258-67.

82. Barbier F, Ruppé E, Hernandez D, et al. Methicillin-resistant coagulase-negative staphylococci in the community: high homology of SCCmec IVa between Staphylococcus epidermidis and major clones of methicillin-resistant Staphylococcus aureus. J Infect Dis 2010; 202: 270-81.

MAILING ADDRESS

Maysa Mandetta Clementino
Instituto Nacional de Controle de Qualidade em Saúde; Fundação Oswaldo Cruz; Av. Brasil, 4365; CEP: 21045-900; Rio de Janeiro-RJ, Brazil; Phone: +55 (21) 3865-5236; Fax: +55 (21) 2290-0915; e-mail: maysa.mandetta@incqs.fiocruz.br.

APÊNDICE D - ARTIGO PUBLICADO NO JOURNAL OF WATER AND HEALTH (2015)

Fecal pollution source tracking in waters intended for human supply based on archaeal and bacterial genetic markers

Kayo Bianco, Camila Barreto, Samara Sant'Anna Oliveira, Leonardo Henriques Pinto, Rodolpho Mattos Albano, Catia Chaia Miranda and Maysa Mandetta Clementino

ABSTRACT

The determination of fecal pollution sources in aquatic ecosystems is essential to estimate associated health risks. In this study, we evaluate eight microbial source tracking (MST) markers including host-specific *Bacteroidales* and *Methanobrevibacter* spp. for discrimination between human, bovine, equine, and swine fecal contamination in waters intended for human supply. Overall, the novel host-specific archaeal and bacterial primers proposed in this study demonstrated high sensitivity and specificity. Markers for the Archaea domain were more prevalent in the fecal and water samples studied. We conclude that the investigations regarding the sources of fecal pollution in public water supplies can contribute to improve the quality of human health. To our knowledge, this is the first analysis using both archaeal and bacterial fecal MST markers on tropical water bodies of Rio de Janeiro city, Brazil.

Key words | *Bacteroidales*, fecal pollution, *Methanobrevibacter* spp., microbial source tracking, water quality

Kayo Bianco
Camila Barreto
Samara Sant'Anna Oliveira
Catia Chaia Miranda
Maysa Mandetta Clementino (corresponding author)
Instituto Nacional de Controle de Qualidade em Saúde, INCQS/FIOCRUZ,
Av. Brasil, 4365 - Manguinhos,
Rio de Janeiro,
RJ,
Brazil
E-mail: *maysa.mandetta@incqs.fiocruz.br*

Leonardo Henriques Pinto
Rodolpho Mattos Albano
Centro Biomédico do Hospital Universitário Antônio Pedro,
Universidade Estadual do Rio de Janeiro, UERJ,
Rua Felipe Camarão, 58 - Vila Isabel,
Rio de Janeiro,
RJ,
Brazil

INTRODUCTION

Concerns about water quality have increased in recent years, partly due to frequent contamination of coastal and inland water resources by sewage carrying waterborne pathogens. Waterborne diseases are mainly caused by enteric pathogenic micro-organisms, which are transmitted primarily by the fecal–oral route (USDA 2012). Consequently, this situation has been aggravated in recent years due to frequent contamination of drinking, recreational, and irrigation waters by emerging pathogens such as *Giardia lamblia*, *Cryptosporidium parvum*, Enterohemorrhagic *Escherichia coli* O157:H7, *Vibrio cholera*, among others (WHO 2006).

Fecal pollution can reach water bodies through discharge of fecal waste or raw sewage, wastewater from livestock, hospitals, slaughterhouses, and industrial activities, among other sources (USEPA 2005). The possibility of animal waste reaching underground sources of drinking water represents a significant public health threat. In addition, aquifers worldwide are experiencing increasing pollution threats from urbanization, industrial development, agricultural activities, and mining enterprises. Groundwater is a vital natural resource for the economic and secure provision of potable water supply in both urban and rural settings (Foster *et al.* 2002).

Owing to the economic development of metropolitan regions, the exploitation of environmental resources impacts on water availability in rivers, reservoirs, and other water sources, both in terms of quantity and quality. Consequently, there is increasing pollution of waterbodies located in the vicinity of urban regions where popular demand for proper pollution control is ever increasing (Gonçalves 2009). Thus, the monitoring of raw water is of fundamental importance in the water treatment station operational

doi: 10.2166/wh.2015.292

routine, since water quality is dynamic in time and space (Carmo *et al.* 2008; Di Bernardo & Paz 2008). This dynamism has a direct influence in water treatment processes, as contaminated waters require complex procedures that generally lead to increases in water prices.

Worldwide, water quality is evaluated using culture-based enumeration of fecal indicator bacteria (e.g., *E. coli*, *enterococci*) and, more recently, by quantitative real-time PCR (USEPA 2012). However, neither of these approaches provide information about the source of fecal pollution since these organisms are normal inhabitants of the gastrointestinal tracts of several mammals, birds, and insects (Whitman *et al.* 2005; Doud & Zurek 2012). This apparent lack of reliability of traditional indicators has driven the development and implementation of complementary indicators to detect fecal pollution in aquatic environments as microbial source tracking (MST) procedures, resulting in a high diversity of microbial biomarkers (Malakoff 2002; Wu *et al.* 2008; Ahmed *et al.* 2013; Harwood *et al.* 2014).

The determination of fecal pollution sources in aquatic ecosystems is essential to estimate associated health risks and to provide measures to remediate polluted waters (Blanch *et al.* 2008). However, field studies for the determination of the origin of fecal pollution using various available microbiological biomarkers have shown that the current methods present limitations, indicating the need for other markers (Blanch *et al.* 2008; McQuaig *et al.* 2009; Ahmed *et al.* 2012).

Anaerobic micro-organisms constitute the major part of human and other animal microflora. Considering their complex nutritional requirements and inability to grow below 30 °C, which limits their survival in extra-enteric environments, anaerobes such as *Bifidobacterium* spp., *Clostridium perfringens*, *Methanobrevibacter* spp., and members of the order *Bacteroidales* have been currently used as alternative microbial indicators of recent fecal contamination (Stewart *et al.* 2003; Bonjoch *et al.* 2004; Ufnar *et al.* 2006). In addition, their presence can be correlated to host-specific sources of fecal pollution (Bernhard & Field 2000; Bower *et al.* 2005; Savichtcheva & Okabe 2006).

The *Bacteroidales* have been proposed as a host-specific fecal biomarker as they are abundant in the feces of many warm-blooded animals, including humans, and do not survive long in the environment (Bernhard & Field 2000; Meays *et al.* 2004; Dick *et al.* 2005). Other good candidates are archaeal representatives of the genus *Methanobrevibacter* (order *Methanobacteriales*) that includes 16 known species. These microbes inhabit the intestinal tract of animals, decaying plants, and anaerobic sludge from wastewater treatment plants. As only a few species occur in more than one host, they could be seen as specific microbial indicators of fecal pollution in environmental samples (Lai *et al.* 2004; Lee *et al.* 2013). Within this context, a variety of other biomarkers have also been proposed to discriminate fecal pollution events in environmental waters (Scott *et al.* 2002; Martellini *et al.* 2005; Lamendella *et al.* 2007; Fremaux *et al.* 2009).

The main objective of this study was to evaluate the presence of fecal host-specific markers in water bodies intended for human consumption of Região dos Lagos, Rio de Janeiro, Brazil. The São João river is the main water body of the São João basin and together with Bacaxá and Capivari rivers flows to Juturnaíba dam that provides water supply throughout the Região dos Lagos (Figure 1). As in many developing countries, large parts of the Brazilian population have no regular access to improved sanitation. This situation is all the more blatant in rural areas. Thus, there is a lack of information about wastewater disposal sources and no quality control data. The suspected sources of fecal pollution in these waterbodies are animal husbandry waste from small farms in their vicinity with direct access to the reservoir, native and feral wildlife and, to a lesser extent, human recreational activities.

In this work, microbial indicators from biologically significant fecal pollution sources such as human, equine, bovine, and swine were implemented. The host-specificity and sensitivity of *Bacteroidales* and *Methanobrevibacter* markers were assessed in fecal samples from target and non-target host groups. Subsequently, environmental water samples from São João basin were evaluated.

METHODS

Fecal sampling

To determine the host-specificity and sensitivity of the markers, 49 fecal samples were collected from six host groups.

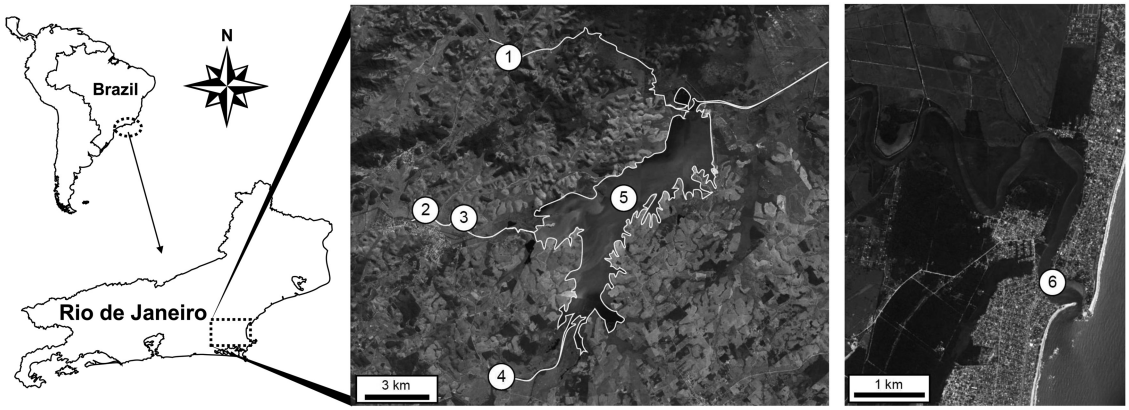


Figure 1 | Geographical location of the São João river basin in the northern state of Rio de Janeiro: (1) São João river, (2) Capivari river, (3) Capivari railway, (4) Bacaxá river, (5) Juturnaíba dam, (6) São João river mouth.

Human feces ($n = 12$) were obtained from volunteers of the laboratory staff. Fecal samples of horse ($n = 12$), pig ($n = 8$), sheep ($n = 3$), chicken ($n = 3$), and cow ($n = 11$) came from two different farms in Mato Grosso do Sul, Brazil. Fresh animal stool samples (2–20 g) were taken in sterile Falcon tubes, maintened on ice and transported to the laboratory. Between 0.3 and 0.5 g of fecal material were resuspended in 1 mL of sodium phosphate buffer (PBS) (0.12 mol L^{-1}, pH 8.0). The diluted fecal mixture was mixed on a rotating platform to produce a homogeneous suspension and stored at −20 °C.

Environmental water sampling

Environmental water samples ($n = 12$) were collected in two different seasons (November 2013 and May 2014) from Juturnaíba dam (22 °38′S/42 °18′W), São João river (22 °35′S/41 °59′), São João river mouth (22 °35′/41 °59′W), Capivari river (22 °38′S/42 °24′), Capivari railway (22 °38′S/42 °22′W) and Bacaxá river (22 °42′S/42 °21′) (Figure 1). Six of the 12 samples were collected in dry weather and the remaining six were collected following wet weather events. The water samples (5.0 L) were taken at a depth of approximately 15–20 cm below the surface in a sterile polyethylene bottle. All samples were stored on ice and conducted to the laboratory within 4 h. The enumeration of *E. coli* in 100 mL was carried out using the defined substrate method (Colilert, IDEXX), according to the protocol described in Standard Methods for the Examination of Water and Wastewater (APHA 2012). Then, 4 L of each water sample were filtered through a 0.22 μm Stericup® system (Millipore). In case of filter clogging, additional filters were added. The filters were placed in 2 mL microcentrifuge tubes with 1 mL of PBS and kept at −20 °C overnight.

DNA extraction

The procedure used for DNA extraction was a modified version of previously described protocols (Ogram *et al.* 1987; Smalla *et al.* 1993). Briefly, tubes containing water sample filters and the homogeneous suspension of fecal samples were submitted to three cycles of freezing and thawing (−70 °C/2 min, 65 °C/2 min). Then, an equal volume of glass beads (0.1 mm diameter) was added and the suspension was shaken three times for 80 s at maximum speed in a Bead-Beater. The liquid phase was extracted with phenol–chloroform [1:1 (v/v)] and chloroform–isoamyl alcohol [24:1 (v/v)]. The DNA was precipitated from the aqueous phase with three volumes of ethanol and after being dried the pellet was resuspended in 100 μL of deionized water. For further purification of the DNA, we used the Dneasy Tissue Kit (Qiagen GmgH, Hilden, Germany) according to the manufacturer's instructions to remove possible PCR inhibitors (Clementino *et al.* 2007). Purified DNA from fecal **Q5** and water samples was quantified in order to standardize the DNA concentration for further PCR reactions. These measures were obtained with a Qubit® 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The possible presence of DNA amplification inhibitors in fecal and water samples was assessed by PCR

reactions targeting *Bacteroidales* 16S rRNA gene with universal primers Bac32F and Bac708R (Bernhard & Field 2000) and *Methanobrevibacter* spp. *mcrA* gene with universal primers mcrAf and mcrAr (Luton *et al.* 2002). *Bacteroides fragilis* INCQS 00068/ATCC 25285 and *Methanobrevibacter smithii* INCQS A45D/DSM 11975 were used as positive control for *Bacteroidales* and *Methanobrevibacter* spp., respectively. *E. coli* INCQS 00043/ATCC 23229, *Klebsiella pneumoniae* INCQS 00629/ATCC BAA-1706, *Pantoea agglomerans* INCQS 00721/ATCC 33243 were used as negative control for both reactions. DNA templates that yielded negative results were diluted 1:10 with sterile distilled H_2O and re-tested. Approximately 25% of all fecal and water samples tested showed initial inhibition before dilution. All samples producing fragments of the expected sizes were further analyzed with the MST markers.

Primer development

PCR reactions for the detection of human- and equine-associated *Bacteroidales* were performed according to previously described protocols (Bernhard & Field 2000; Dick *et al.* 2005). However, there were no amplified fragments of the expected size even after several optimization procedures. To circumvent this issue, we designed new equine and human *Bacteroidales* specific oligonucleotides based on 16S rRNA sequences (AY212554 and AF233411, respectively) available in public databases. We also designed a new primer set for the detection of *M. gottschalkii* based on the *mcrA* gene (EU919431). The primers were designed using Primer-BLAST software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and synthesized by Invitrogen (Carlsbad, CA, USA). Primer specificity analysis was done *in silico* using BLASTn against the NCBI database and *In Silico*-PCR amplification against archaeal and bacterial genomic DNA (http://insilico.ehu.es/PCR).

Specificity, sensitivity, and limit of detection

MST primer sensitivity (r) and specificity (s) were tested using DNA from human, pig, horse, cow, sheep, and chicken feces. The r and s values were calculated according to the following formulas: $r = [TP/(TP + FN)]$ and $s = [TN/(TN + FP)]$, where TP is the number of samples that were positive for the PCR marker of their own species (true positive); FN is the number of samples that were negative for a PCR marker of their own species (false negative); TN is the number of samples that were negative for a PCR marker of another species (true negative); and FP is the number of samples that were positive for a PCR marker of another species (false positive).

To determine the limit of detection (LOD) of the equine- and human-associated *Bacteroidales* and equine-associated *Methanobrevibacter* markers, serial dilutions (10^{-1}–10^{-11}) from 20 ng of fecal DNA samples were made.

PCR conditions

PCR analysis was carried out in 50 μL amplification reaction mixtures containing 1 × PCR buffer (Invitrogen), 5% dimethyl sulfoxide (w/v), 200 μmol L^{-1} dNTPs (Invitrogen), 2 U of Platinum *Taq* DNA Polymerase (Invitrogen), 1 pmol of each primer, optimum $MgCl_2$ concentration (Table 1) and about 20 ng of DNA template. The cycling conditions consisted of an initial 95 °C step for 5 min and 40 cycles of amplification at 95 °C for 1 min, an annealing temperature specific for each primer set (Table 1) for 1 min, 72 °C for 1 min and a final elongation at 72 °C for 6 min. PCR products were loaded onto a 1% (v/v) agarose gel, and were separated by electrophoresis at 70 V for 2 h in 1 × TAE (40 mmol L^{-1} Tris base, 20 mmol L^{-1} sodium acetate, 1 mmol L^{-1} EDTA, pH 8.0) buffer with a 100 bp DNA ladder (Invitrogen) as molecular weight standard. The gels were stained with ethidium bromide and gel images were digitalized with the Video Documentation System and analyzed with Image-Master software (Amersham Pharmacia Biotech).

The reproducibility for amplification of host-specific *Bacteroidales* and archaeal markers was evaluated in triplicate PCR reactions with at least two fecal DNA samples from target hosts. In addition, for archaeal markers, DNA from the reference strains *Methanobrevibacter smithii* INCQS A45D/DSM 11975, *M. ruminantium* INCQS A36D/DSM 1093, and *M. gottschalkii* INCQS A49D/DSM 11977 were used as positive controls for human, bovine, and equine MST markers, respectively.

Host-specific *Bacteroidales* PCR fragments were purified using the QIAquick® PCR Purification kit (Qiagen GmgH, Hilden, Germany) and sequenced with the Big

Table 1 | Oligonucleotides, target genes, and PCR conditions

Primer	Sequence (5′-3′)	Target	MgCl2 (mM)	Annealing temp (°C)	Product size (bp)	Reference	
HuM113F	ACTCTTGGCCAGCCTTCTGA	16S rRNA/Human-associated *Bacteroidales*	1.5	57	290	This study	
HuM403R	ACCCATAGGGCAGTCATCCT						
Mnif-342F	AACAGAAAACCCAGTGAAGAG	*nifH*/*Methanobrevibacter smithii*	2.0	58	222	Ufnar (2005)	Q11
Mnif-363R	ACGTAAAGGCACTGAAAAACC						
CF128F	CCAACYTTCCCGWTACTC	16S rRNA/Bovine-associated *Bacteroidales*	1.5	56	464	Bernhard & Field (2000)	
CF592R	AYMTCCCGTCTACGCTCC					Liu (2012)	Q12
Mrnif-F	AATATTGCAGCAGCTTACAGTGAA	*nifH*/*Methanobrevibacter ruminantium*	2.0	56	336	Ufnar (2007a)	Q13
Mrnif-R	TGAAAATCCTCCGCAGACC						
PF163F	GCGGATTAATACCGTATGA	*16S rRNA*/Swine-associated *Bacteroidales*	1.5	55	385	Dick (2005)	Q14
PF548R	CCCAATAAATCCGGATAACG					This study	
P23-2 F	TCTGCGACACCGGTAGCCATTGA	*mcrA*/P23-2 Clone	2.0	60	258	Ufnar (2007b)	Q15
P23-2 R	ATACACTGGCGACATTCTTGAGGATTAC						
HoR201F	TGGGGATGCGTCTGATTAGC	*16S rRNA*/Equine-associated *Bacteroidales*	1.5	53	242	This study	
HoR442R	CCCACACGTGGGTCACTTTA						
GoT285F	GCACAAACTGGTTTAAGCGGA	*mcrA*/*Methanobrevibacter gottschalkii*	1.5	54	120	This study	
GoT404R	GGAGAATACGTTAGCAGCACCA						

Dye Terminator Kit and analyzed on the ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA). Electropherograms were converted to fasta format through Sequencher 3.0 software (Gene Codes Corporation, Ann Harbor, MI, USA). Nucleotide similarity searches were carried out online with BLASTn (http://www.ncbi.nlm.nih.gov/BLAST/) against GenBank (NCBI). The sequences reported in this study were submitted to GenBank/NCBI database under accession numbers KM924823–KM924826.

RESULTS

Enumeration of *E. coli*

Ten water samples showed *E. coli* levels within acceptable limits (0–920 MPN/100 mL) according to Brazilian standards (CONAMA 2005). The two other samples, Capivari railway and São João river mouth, had *E. coli* counts above the recommended limit (1,119.9 and 2,682 MPN/100 mL, respectively).

Specificity, sensitivity, and LOD

Host-specificity and -sensitivity of human-, bovine-, equine-, and swine-associated markers were evaluated by screening 49 fecal samples from six host groups. The archaeal human marker was detected in all (12/12) human fecal DNA samples tested but not in non-target host groups (0/37). However, the *Bacteroidales* human marker was positive in 8 of 12 human samples and 1 of 37 animal fecal DNA samples (Table 2). The bovine archaeal marker was positive for 10 of 11 bovine DNA samples while the *Bacteroidales* bovine marker was detected in all cow fecal samples analyzed

Table 2 | Specificity assays for host-specific markers in fecal samples

		No. of positive PCR results							
			Human markers		Bovine markers		Swine markers		Equine markers
Target	No. of samples tested	Archaea	Bacteria	Archaea	Bacteria	Archaea	Bacteria	Archaea	Bacteria
Human	12	12/12 (100%)	8/12 (67%)	ND	ND	ND	ND	ND	ND
Swine	8	ND	ND	ND	ND	8/8 (100%)	7/8 (91%)	ND	ND
Cow	11	ND	ND	(10/11) 91%	11/11 (100%)	ND	ND	ND	ND
Horse	12	ND	ND	ND	ND	ND	ND	12/12 (100%)	10/12 (83%)
Chicken	3	ND	ND	ND	ND	ND	ND	ND	ND
Sheep	3	ND	1/3 (33%)	ND	ND	ND	ND	ND	ND

ND, not detected.

(11/11). The swine archaeal marker was detected in all pig fecal samples (8/8) while the archaeal equine marker was amplified in all equine samples (12/12). On the other hand, the swine and equine *Bacteroidales* markers were detected in 7 of 8 and 10 of 12 of each host's samples, respectively (Table 2).

The overall sensitivity of the human-, swine-, and equine-associated archaeal markers to differentiate between its own host group and other animal fecal samples was 1 (maximum value of 1) as was the bovine-associated bacterial marker. Human-, equine-, and swine-associated *Bacteroidales* indicator sensitivity values were 0.67, 0.83, and 0.88, respectively, whereas for the bovine-associated archaeal marker it was 0.91. The specificity value for all archaeal markers and for swine- and equine-associated *Bacteroidales* markers was 1 (maximum value of 1). The specificity value for both human and bovine *Bacteroidales* markers was 0.97. All sensitivity and specificity results are given in Table 3.

The equine archaeal marker was able to amplify a visible fragment up to a dilution of 10^{-5} of horse fecal DNA. The LOD of the remaining biomarkers was stablished at a 10^{-2} DNA dilution. To determine the reproducibility of the assays, several replicates ($n = 5$) of serially diluted genomic DNA were tested.

Biomarker detection in water samples

M. smithii and *M. ruminantium* were detected in all (12/12) water samples while human- and ruminant-associated *Bacteroidales* markers were detected in 9 and 8 of 12 samples, respectively. The P23-2 clone (*mcrA* gene) was present in 8 of 12 water samples and swine-associated *Bacteroidales* was present in all samples (12/12). The *M. gottschalkii* marker was found in 12 of 12 water samples, while the equine-associated *Bacteroidales* was detected in 7 of 12 samples analyzed (Figure 2).

Table 3 | Sensitivity and specifity of the MST markers

	Human markers		Bovine markers		Swine markers		Equine markers	
Value	Archaea	Bacteria	Archaea	Bacteria	Archaea	Bacteria	Archaea	Bacteria
Sensitivity (r)	1	0.67	0.91	1	1	0.88	1	0.83
Specificity (s)	1	0.97	1	0.97	1	1	1	1

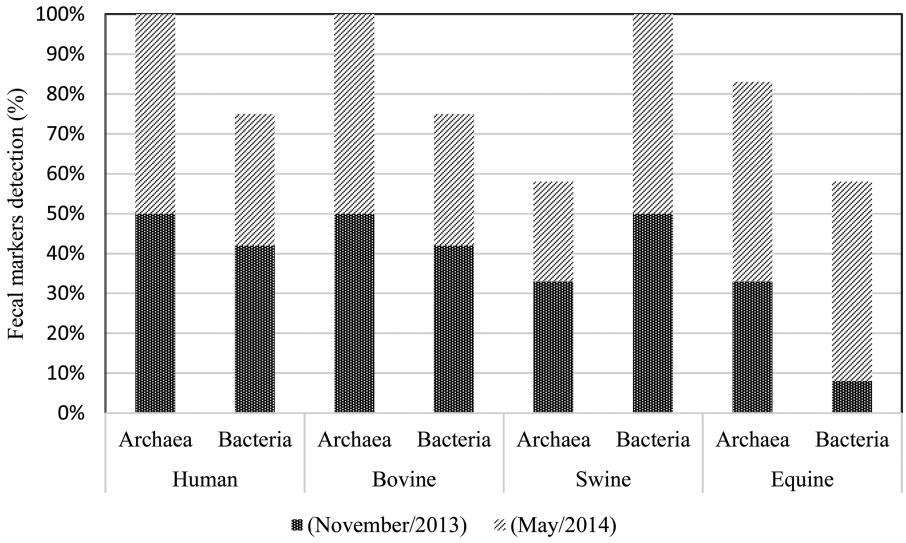


Figure 2 | Detection of biomarkers for different fecal sources.

16S rRNA sequences were analyzed by BLAST to search for sequence similarity with other sequences already available in the GenBank database. The 16S rRNA gene sequences of *Bacteroidales* markers showed 98% identity with those of bovine, human, and equine and 100% with porcine *Bacteroidales* sequences deposited in GenBank.

DISCUSSION

The aim of this study was to evaluate the presence of fecal host-specific markers in water bodies intended for human consumption. In order to increase the likelihood of identifying fecal contamination sources, novel specific molecular markers, including human-, swine-, and equine-associated *Bacteroidales* and *M. gottschalkii* were proposed (Table 1). In addition, previously described bovine-associated *Bacteroidales* and *M. smithii*, *M. ruminantium*, and swine-associated P23 clone markers were also used (Bernhard Q7 *et al.* 2000; Ufnar *et al.* 2006, 2007a, 2007b; Liu *et al.* 2012). To the best of our knowledge, this is the first analysis using both archaeal and bacterial fecal biomarkers on tropical water bodies of Rio de Janeiro city, Brazil.

The correlation between some MST markers and traditional fecal indicators is not well documented. A study in northwest France showed a significant correlation between *E. coli* concentrations and the presence of human markers whereas no correlation was observed for other animal markers. Some inconsistencies between *E. coli* concentrations and some host-specific *Bacteroidales* in environmental samples have been found previously (Shanks *et al.* 2006; Gourmelon *et al.* 2007). In the present study, all 10 samples presenting *E. coli* levels that were within acceptable limits according to Brazilian Standards (<1,000 MPN/100 mL), showed fecal contamination with at least five MST biomarkers. However, a sample with low and two others with high *E. coli* counts showed the presence of all eight host-specific markers.

Many hypotheses could explain a lack of correlation in some situations: differential fecal inputs, persistence and survival of *E. coli*, methanogens, and *Bacteroidales* in the environment and differences in detection methods, including molecular techniques for host-specific markers and culture for *E. coli* and enterococci (Brion *et al.* 2002; Cole *et al.* 2003; Shanks *et al.* 2006). To identify the origin of fecal contamination, which is currently determined by *E. coli* or enterococcus counts, more information is needed about the persistence of the biomarkers in the environment and their correlation to these fecal indicator bacteria (Long & Sobsey 2004; Shanks *et al.* 2006).

M. smithii and *M. ruminantium nifH* genes were detected in 100% (12/12) of the water samples while human and bovine *Bacteroidales* 16S rDNA sequences were detected in 75% (9/12) of the samples tested. In

addition, the human-associated archaeal marker showed higher specificity and sensitivity than the bacterial one. On the other hand, the bovine-associated archaeal marker showed higher specificity and lower sensitivity compared with the bacterial marker (Table 3).

A study conducted in Australia evaluated the host specificity and sensitivity of the *nifH* gene marker in fecal and wastewater samples from 11 animal species, including humans. The host specificity reported in this particular study was 96% while sensitivity of the marker for human-derived sewage was 81%. The prevalence of the marker in environmental water samples was relatively low compared with the others tested (*esp*, HF183, HPyVs, and adenoviruses). The authors concluded that this marker alone might not be sensitive enough to detect fecal pollution in environmental waters; however, its relatively high host specificity argues for its use in conjunction with other human markers (Ahmed *et al.* 2012). Nevertheless, we observed 100% of sensitivity and specificity for the human host *nifH* marker in our study, supporting its use in future investigations.

Our results also demonstrated that swine fecal contamination was observed in 100% (12/12) of the water samples by the amplification of the *Bacteroidales* marker compared to 66.6% (8/12) detected by the *mcrA* gene from methanogenic organisms. Nevertheless, the *mcrA* gene marker was more sensitive and specific than the swine-associated *Bacteroidales* marker. It is noteworthy that the equine-associated *mcrA* primers described in this study showed high specificity and sensitivity, being detected in 100% (12/12) of the water samples tested in contrast to 58.3% (7/12) using equine-associated *Bacteroidales* markers.

The environmental pollution caused by swine waste is a serious problem due to the high number of contaminants, causing a powerful degradation of air, soil, and mostly of water resources (surface and groundwater) (Seganfredo 2000). Pig waste has a high concentration of biodegradable matter, pathogenic micro-organisms (*Salmonella* spp., *Campylobacter* spp., *Giardia lamblia*, *Taenia solium*), nitrogen, and minerals such as copper, zinc, and arsenic (Schmidt *et al.* 2007; Scanlon *et al.* 2013). In agricultural areas, and even urban areas, we can also observe equine husbandry and horse-breeding, producing about 7–8 kg/animal/day of waste (20% urine and 80% solid material), that is richer in nitrogen than pig or cow feces (Matos 2005). Infections of humans with *Bacillus anthracis* and *Salmonella enterica* that cause anthrax and salmonellosis, respectively, and worms such as *Trichinella spirallis* have been associated with contaminated horse feces (Molinaro 2009).

Despite the importance of the São João river basin, little is known about its water body quality, where monitoring only started in May 2011. Moreover, there are no records of Juturnaíba dam water quality control. Although the dam is considered a wildlife sanctuary, the results of our study demonstrated the presence of fecal pollution from human, bovine, swine, and equine sources.

The novel archaeal and bacterial primers proposed in this study demonstrated high sensitivity and specificity. Interestingly, markers of the Archaea domain were more prevalent, in both the feces and water bodies studied. Nevertheless, a study in northwest France showed that host-specific *Bacteroidales* markers were found to be more sensitive than other biomarkers tested in environmental water samples, especially where low numbers of *E. coli* were found (Gourmelon *et al.* 2007).

Specificity and sensitivity of host-associated markers may vary according to the studied region and should be locally validated before their inclusion in regular water. Variations may occur due to diet or feeding differences and decay rates of the markers at different environmental conditions (Walters & Field 2006; Field & Samadpour 2007; Eichmiller *et al.* 2014). The bovine-associated marker **Q8** *Bacteroidales* CF128, for instance, has been detected in $\leq$10% of pig feces samples in Oregon and Ireland but was observed in $\geq$90% of pig feces samples in France, Portugal, and the UK (Bernhard & Field 2000; Gawler *et al.* 2007).

The MST approach should be carried out using several water samples, with regular sampling intervals combined with conventional fecal indicator monitoring and also include samples taken in both dry and rainfall weather events. Our data suggest that the use of more than one MST marker to identify the source of fecal contamination is valuable because each of these methods has its strengths and weaknesses that can limit the usefulness of MST. The most commonly used marker, for example, *Bacteroides* HF183, is not entirely specific for human waste. However, it has the advantages of being widely distributed in the human population and being present at relatively high

concentration in wastewater (Carson *et al.* 2005; Gawler *et al.* 2007; Gourmelon *et al.* 2007; Ahmed *et al.* 2009).

Finally, we conclude that although MST tools are widely accepted as alternative methods to evaluate sources of pollution, threshold values have not yet been entirely determined to assess the microbial quality of a water body. Meanwhile, the investigations regarding the sources of fecal pollution in public water supplies can contribute to the implementation of better monitoring programs and remediation strategies in order to improve the quality of human health and ecosystems.

ACKNOWLEDGEMENTS

We gratefully acknowledge the Genome Sequencing Core-PDTIS/FIOCRUZ. We also would like to thank Dr Ivano de Filippis for his critical reading of the manuscript. This work was supported by Coordenação de Aperfeiçoamento de Pessoal de Ensino Superior (CAPES), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação Carlos Chagas Filho de Amparo a Pesquisa do Estado do Rio de Janeiro (FAPERJ), and INCQS/FIOCRUZ.

REFERENCES

Ahmed, W., Goonetilleke, A., Powell, D., Chauhan, K. & Gardner, T. 2009 Comparison of molecular markers to detect fresh sewage in environmental waters. *Water Res.* **43** (19), 4908–4917.

Ahmed, W., Sidhu, J. P. & Toze, S. 2012 Evaluation of the *nifH* gene marker of *Methanobrevibacter smithii* for the detection of sewage pollution in environmental waters in Southeast Queensland, Australia. *Environ. Sci. Technol.* **46** (1), 543–550.

Ahmed, W., Sritharan, T., Palmer, A., Sidhu, J. P. S. & Toze, S. 2013 Evaluation of bovine feces-associated microbial source tracking markers and their correlations with fecal indicators and zoonotic pathogens in a Brisbane, Australia, reservoir. *Appl. Environ. Microbiol.* **79** (8), 2682–2691.

APHA (American Public Health Association), AWWA (American Water Works Association), WEF (Water Environment Federation) 2012 *Standard Methods for the Examination of Water and Wastewater*, 22nd edn. APHA, Washington, DC.

Bernhard, A. E. & Field, K. G. 2000 Identification of non-point sources of fecal pollution in coastal waters by using host-specific 16S ribosomal DNA genetic markers from fecal anaerobes. *Appl. Environ. Microbiol.* **66** (4), 1587–1594.

Blanch, A. R., Lucena, F., Payán, A. & Jofre, J. 2008 Minimal requirements for parameters to be used in the development of predictive models for microbial source tracking: somatic coliphages and phages infecting *Bacteroides* as examples. *J. Environ. Detect.* **1** (1), 2–21.

Bonjoch, X., Balleste, E. & Blanch, A. R. 2004 Multiplex PCR with 16S rRNA gene targeted primers of *Bifidobacterium* spp. to identify sources of fecal pollution. *Appl. Environ. Microbiol.* **70** (5), 3171–3175.

Bower, P. A., Scopel, C. O., Jensen, E. T., Depas, M. M. & Mclellan, S. L. 2005 Detection of genetic markers of fecal indicator bacteria in Lake Michigan and determination of their relationship to *Escherichia coli* densities using standard microbiological methods. *Appl. Environ. Microbiol.* **71** (12), 8305–8313.

Brion, G. M., Meschke, J. S. & Sobsey, M. D. 2002 F-specific RNA coliphages: occurrence, types, and survival in natural waters. *Water Res.* **36** (9), 2419–2425.

Byamukama, D., Kansiime, F., Mach, R. L. & Farneleither, H. 2000 Determination of *Escherichia coli* contamination with chromocult coliform agar showed a high level of discrimination efficiency for differing fecal pollution levels in tropical water of Kampala, Uganda. *Appl. Environ. Microbiol.* **66** (2), 864–868. Q9

Carmo, R. F., Bevilacqua, P. D. & Bastos, R. K. X. 2008 Drinking water quality surveillance: qualitative approach of hazard identification. *Eng. Sanit. Ambient.* **13** (4), 426–434.

Carson, C. A., Christiansen, J. M., Yampara-Iquise, H., Benson, V. W., Baffaut, C., Davis, J. V., Broz, R. R., Kurtz, W. B., Rogers, W. M. & Fales, W. H. 2005 Specificity of a *Bacteroides thetaiotaomicron* marker for human feces. *Appl. Environ. Microbiol.* **7** (8), 4945–4949.

Clementino, M. M., Fernandes, C. C., Vieira, R. P., Cardoso, A. M., Polycarpo, C. R. & Martins, O. B. 2007 Archaeal diversity in naturally occurring and impacted environments from a tropical region. *J. Appl. Microbiol.* **103** (1), 141–151.

Cole, D., Long, S. C. & Sobsey, M. D. 2003 Evaluation of F+ RNA and DNA coliphages as source-specific indicators of fecal contamination in surface waters. *Appl. Environ. Microbiol.* **69** (11), 6507–6514.

CONAMA (Brazilian National Environmental Council) 2005 *Resolution n. 357: On the classification of water bodies and environmental guidelines for its categorization, as well as it establishes conditions and standards for effluent release, and provides other measures*. DOU n. 053, pp. 58–63.

Di Bernardo, L. & Paz, L. P. S. 2008 *Selection of Water Treatment Technology*, Vol. 1, 1st edn. LDiBe, São Paulo, Brazil.

Dick, L. K., Bernhard, A. E., Brodeur, T. J., Santo Domingo, J. W., Simpson, J. M., Walters, S. P. & Field, K. G. 2005 Host distributions of uncultivated fecal *Bacteroidales* bacteria reveal genetic markers for fecal source identification. *Appl. Environ. Microbiol.* **71** (6), 3184–3191.

Doud, C. W. & Zurek, L. 2012 Enterococcus faecalis OG1RF: pMV158 survives and proliferates in the house fly digestive tract. *J. Med. Entomol.* **49** (1), 150–155.

Eichmiller, J. J., Bajer, P. G. & Sorensen, P. W. 2014 The relationship between the distribution of common carp and their environmental DNA in a small lake. *PLoSOne.* **9** (11), e112611.

Field, K. G. & Samadpour, M. 2007 Fecal source tracking, the indicator paradigm, and managing water quality. *Water Res.* **41** (16), 3517–3538.

Foster, S., Hirata, R., Gomes, D., D'elia, M. & Paris, M. 2002 *Groundwater Quality Protection: A Guide for Water Service Companies, Municipal Authorities and Environment Agencies*. The World Bank, Washington, DC, p. 114.

Fremaux, B., Gritzfeld, J., Boa, T. & Yost, C. K. 2009 Evaluation of host-specific *Bacteroidales* 16S rRNA gene markers as a complementary tool for detecting fecal pollution in a prairie watershed. *Water Res.* **43** (19), 4838–4849.

Gawler, A. H., Beecher, J. E., Brandão, J., Carroll, N. M., Falcão, L., Gourmelon, M., Masterson, B., Nunes, B., Porter, J., Rince, A., Rodrigues, R., Thorp, M., Walters, J. M. & Meijer, W. G. 2007 Validation of host-specific *Bacteroidales* 16S rRNA genes as markers to determine the origin of faecal pollution in Atlantic Rim countries of the European Union. *Water Res.* **41** (16), 3780–3784.

Gonçalves, R. F. 2009 *Water and Energy Conservation in Private and Public Systems of Water Supply*. ABES, Rio de Janeiro, Brazil.

Gourmelon, M., Caprais, M. P., Segura, R., Le Mennec, C., Lozach, S., Piriou, J. Y. & Rince, A. 2007 Evaluation of two library independent microbial source tracking methods to identify sources of fecal contamination in French estuaries. *Appl. Environ. Microbiol.* **73** (15), 4857–4866.

Harwood, V. J., Staley, C., Badgley, B. D., Borges, K. & Korajkic, A. 2014 Microbial source tracking markers for detection of fecal contamination in environmental waters: relationships between pathogens and human health outcomes. *FEMS Microbiol. Rev.* **38** (1), 1–40.

Lai, M. C., Lin, C. C., Yu, P. H., Huang, Y. F. & Chen, S. C. 2004 *Methanocalculus chunghsingensis* sp. nov., isolated from an estuary and a marine fishpond in Taiwan. *Int. J. Syst. Evol. Microbiol.* **54** (1), 183–189.

Lamendella, R., Domingo, J. W. S., Oerther, D. B., Vogel, J. R. & Stoeckel, D. M. 2007 Assessment of fecal pollution sources in a small northern-plains watershed using PCR and phylogenetic analyses of Bacteroidetes 16S rRNA gene. *FEMS Microbiol. Ecol.* **59** (3), 651–660.

Lee, J. H., Kumar, S., Lee, G. H., Chang, D. H., Rhee, M. S., Yoon, M. H. & Kim, B. C. 2013 *Methanobrevibacter boviskoreani* sp. nov., isolated from the rumen of Korean native cattle. *Int. J. Syst. Evol. Microbiol.* **63** (11), 4196–4201.

Liu, R., Chan, C. F., Lun, C. H. & Lau, S. C. 2012 Improving the performance of an end-point PCR assay commonly used for the detection of *Bacteroidales* pertaining to cow feces. *Appl. Microbiol. Biotechnol.* **93** (4), 1703–1713.

Long, S. C. & Sobsey, M. D. 2004 A comparison of the survival of F + RNA and F + DNA coliphages in lake water microcosms. *J. Water Health.* **2** (1), 15–22.

Luton, P. E., Wayne, J. M., Sharp, R. J. & Riley, P. W. 2002 The *mcrA* gene as an alternative to 16S rRNA in the phylogenetic analysis of methanogen populations in landfill. *Microbiology* **148** (11), 3521–3530.

Malakoff, D. 2002 Microbiologists on the trail of polluting bacteria. *Science.* **295** (5564), 2352–2353.

Martellini, A., Payment, P. & Villemur, R. 2005 Use of eukaryotic mitochondrial DNA to differentiate human, bovine, porcine and ovine sources in fecally contaminated surface water. *Water Res.* **39** (4), 541–548.

Matos, A. T. 2005 *Management and Treatment of Agroindustrial Waste*. (Series. Didactic Notebooks No. 31). AEAGRI, Viçosa, p. 128.

McQuaig, S. M., Scott, T. M., Lukasik, J. O., Paul, J. H. & Harwood, V. J. 2009 Quantification of human polyomaviruses JC Virus and BK Virus by TaqMan quantitative PCR and comparison to other water quality indicators in water and fecal samples. *Appl. Environ. Microbiol.* **75** (11), 3379–3388.

Meays, C. L., Broersma, K., Nordin, R. & Mazumder, A. 2004 Source tracking fecal bacteria in water: a critical review of current methods. *J. Environ. Manage.* **73** (1), 71–79.

Molinaro, E. M. 2009 *Methods and Procedures of Training for Health Laboratory Professionals*, Vol. 4. EPSJV/IOC, Rio de Janeiro, Brazil.

Ogram, A., Sayler, G. S. & Barkay, T. 1987 The extraction and purification of microbial DNA from sediments. *J. Microbiol. Methods* **7**, 57–66.

Savichtcheva, O. & Okabe, S. 2006 Alternative indicators of fecal pollution: relations with pathogens and conventional indicators, current methodologies for direct pathogen monitoring and future application perspectives. *Water Res.* **40** (13), 2463–2476.

Scanlon, K. A., Cagney, C., Walsh, D., Mcnulty, D., Carroll, A., Mcnamara, E. B., Mcdowell, D. A. & Duffy, G. 2013 Occurrence and characteristics of fastidious Campylobacteraceae species in porcine samples. *Int. J. Food Microbiol.* **163** (1), 6–13.

Schmidt, G. A., Legrande, A. N. & Hoffmann, G. 2007 Water isotope expressions of intrinsic and forced variability in a coupled ocean-atmosphere model. *J. Geophys. Res.* **112** (D10), D10103, doi: 10.1029/2006JD007781.

Scott, T. M., Rose, J. B., Jenkins, T. M., Farrah, S. R. & Luhasik, J. 2002 Microbial source tracking: current methodology and future directions. *Appl. Environ. Microbiol.* **68** (12), 5796–5803.

Seganfredo, M. A. 2000 *The Environmental Issue in the use of pig Manure as a Soil Fertilizer, Technical Circular Letter 22*. Embrapa Swine and Poultry, Santa Catarina, Brazil.

Shanks, O. C., Nietch, C., Simonich, M., Younger, M., Reynolds, D. & Field, K. G. 2006 Basin-wide analysis of the dynamics of fecal contamination and fecal source identification in

Tillamook Bay, Oregon. *Appl. Environ. Microbiol.* **72** (8), 5537–5546.

Smalla, K., Cresswell, N., Mendonca-Hagler, L. C., Wolters, A. & van Elsas, J. D. 1993 Rapid DNA extraction protocol from soil for polymerase chain reaction-mediated amplification. *J. Appl. Bacteriol.* **74**, 78–85.

Stewart, J. R., Ellender, R. D., Gooch, J. A., Jiang, S., Myod, P. & Weisberg, S. B. 2003 Recommendations for microbial source tracking: lessons from a methods comparison study. *J. Water Health* **1** (4), 225–231.

Ufnar, J. A., Wang, S. Y., Christiansen, J. M., Yampara-Iquise, H., Carson, C. A. & Ellender, R. D. 2006 Detection of the *nifH* gene of *Methanobrevibacter smithii*: a potential tool to identify sewage pollution in recreational waters. *J. Appl. Microbiol.* **101** (1), 44–52.

Ufnar, J. A., Wang, S. Y., Ufnar, D. F. & Ellender, R. D. 2007a *Methanobrevibacter ruminantium* as an indicator of domesticated-ruminant fecal pollution in surface waters. *Appl. Environ. Microbiol.* **73** (21), 7118–7121.

Ufnar, J. A., Ufnar, D. F., Wang, S. Y. & Ellender, R. D. 2007b Development of a swine specific fecal pollution marker based on host differences in methanogen *mcrA* genes. *Appl. Environ. Microbiol.* **73** (16), 5209–5217.

USDA 2012 *Introduction to Waterborne Pathogens in Agricultural Watersheds*. United States Department of Agriculture, Washington, DC.

USEPA 2005 *Microbial Source Tracking Guide Document, EPA 600-R-05-064*. Office of Research and Development, United States Environmental Protection Agency, Washington, DC.

USEPA 2012 *Method 1611: Enterococci in Water by TaqMan® Quantitative Polymerase Chain Reaction (qPCR) Assay, EPA-821-R-12-008*. Office of Water, United States Environmental Protection Agency, Washington, DC.

USEPA 2014 *The Water Quality Standards HandBook*. EPA/40 CFR part 131. United States Environmental Protection Agency, Office of Research and Development, Cincinatti, OH. Q10

Walters, S. P. & Field, K. G. 2006 Persistence and growth of fecal *Bacteroidales* assessed by bromodeoxyuridine immunocapture. *Appl. Environ. Microbiol.* **72** (7), 4532–4539.

Whitman, R. L., Byers, S. E., Shively, D. A., Ferguson, D. M. & Byappanahalli, M. 2005 Occurrence and growth characteristics of *Escherichia coli* and enterococci within the accumulated fluid of the northern pitcher plant (*Sarracenia purpurea* L.). *Can. J. Microbiol.* **51** (12), 1027–1037.

WHO 2006 *Guidelines for Drinking Water Quality: Incorporating First Addendum*, Vol. 1, 3rd edn. World Health Organization, Geneva.

Wu, J., Yue, J., Hu, R., Yang, Z. & Zhang, L. 2008 Use of caffeine and human pharmaceutical compounds to identify sewage contamination. *World Acad. Sci. Eng. Technol.* **44**, 438–442.

First received 22 December 2014; accepted in revised form 10 April 2015. Available online 1 June 2015

APÊNDICE E - ARTIGO PUBLICADO NA PLOS ONE (2012)

OPEN ACCESS Freely available online

PLOS ONE

Pollution Impacts on Bacterioplankton Diversity in a Tropical Urban Coastal Lagoon System

Gigliola R. B. Salloto[1], Alexander M. Cardoso[2]*, Felipe H. Coutinho[3], Leonardo H. Pinto[3], Ricardo P. Vieira[3], Catia Chaia[1], Joyce L. Lima[3], Rodolpho M. Albano[4], Orlando B. Martins[3], Maysa M. Clementino[1]

1 Laboratório de Microrganismos de Referência, Instituto Nacional de Controle de Qualidade em Saúde, Fundação Oswaldo Cruz, Rio de Janeiro, Brazil, **2** Diretoria de Programa, Instituto Nacional de Metrologia, Qualidade e Tecnologia, Rio de Janeiro, Brazil, **3** Instituto de Bioquímica Médica, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil, **4** Departamento de Bioquímica, Universidade do Estado do Rio de Janeiro, Rio de Janeiro, Brazil

Abstract

Despite a great number of published studies addressing estuarine, freshwater and marine bacterial diversity, few have examined urban coastal lagoons in tropical habitats. There is an increasing interest in monitoring opportunistic pathogens as well as indigenous microbial community members in these water bodies by current molecular and microbiological approaches. In this work, bacterial isolates were obtained through selective plate dilution methods to evaluate antibiotic resistances. In addition, 16S rRNA gene libraries were prepared from environmental waters and mixed cultures grown in BHI medium inoculated with Jacarepaguá lagoon waters. Denaturing gradient gel electrophoresis (DGGE) analyses showed distinct community profiles between environmental communities from each studied site and their cultured counterparts. A total of 497 bacterial sequences were analyzed by MOTHUR, yielding 245 operational taxonomic units (OTUs) grouped at 97% similarity. CCA diagrams showcased how several environmental variables affect the distribution of 18 bacterial orders throughout the three distinct habitats. UniFrac metrics and Venn diagrams revealed that bacterial communities retrieved through each experimental approach were significantly different and that only one OTU, closely related to *Vibrio cholerae*, was shared between them. Potentially pathogenic bacteria were isolated from most sampled environments, fifty percent of which showed antibiotic resistance.

Citation: Salloto GRB, Cardoso AM, Coutinho FH, Pinto LH, Vieira RP, et al. (2012) Pollution Impacts on Bacterioplankton Diversity in a Tropical Urban Coastal Lagoon System. PLoS ONE 7(11): e51175. doi:10.1371/journal.pone.0051175

Editor: David L. Kirchman, University of Delaware, United States of America

Received June 19, 2012; **Accepted** October 30, 2012; **Published** November 30, 2012

Funding: Financial support was provided by PETROBRAS, Foundation for Research Support of the State of Rio de Janeiro, Oswaldo Cruz Foundation and National Council for Scientific and Technological Development. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors received funding from PETROBRAS. There are no patents, products in development or marketed products to declare. This does not alter the authors' adherence to all the PLOS ONE policies on sharing data and materials.

* E-mail: amcardoso@bioqmed.ufrj.br

Introduction

Urban coastal lagoons are impacted environments that are highly affected by mixing of sediments, seawater, and continental freshwater. These aquatic bodies offer aesthetic, economic and recreational value and also function as catchments for stormwater runoff. Furthermore, in tropical regions reminiscent natural mangrove habitats provide protection, food and breeding areas for different animal and plant species [1].

Coastal urban lagoons are subjected to anthropogenic impacts that generate high sedimentation rates and eutrophication [2]. Their maintenance as functional and healthy ecosystems is essential for our future welfare [3]. According to the World Health Organization (WHO), 4.0% of all deaths and 5.7% of the global disease burden are due to water related illnesses, stemming from poor water quality, hygiene and sanitation [4]. Pollution of water bodies with feces originating from different sources could lead to pathogen transmission and, therefore, to waterborne diseases [5].

Indicator bacteria, including total and fecal coliform and *Enterococci*, have been widely used as a monitoring tool for microbial contamination of water [6]. Although their presence provides information regarding health risks associated to gastrointestinal pathogens, this indicator system does not predict specific pathogens or sources of fecal contamination [7]. Fecal pollution in aquatic environments can lead to serious consequences when enteric pathogens are present. Access to both clean water and sanitation is not widespread in developing countries. According to WHO, more than 50% of water associated diseases are microbial intestinal infections, causing at least 11,399 deaths worldwide due to cholera [8].

As human populations increase, anthropogenic impacts affect lakes, rivers and coastal ecosystems mainly due to discharge of sewage and chemical compounds such as pesticides, hormones and antibiotics that are largely used in human and veterinary clinics [9]. The main source of antibiotics in aquatic environments comes from wastewater discharge, manure disposal and aquaculture [10]. Furthermore, their indiscriminate use has accelerated the spread of antibiotic resistance genes. In addition, antibiotics are often produced by environmental microorganisms that harbor intrinsic resistance genes [11]. Aquatic environments are sites of intense genetic exchange, in which originally susceptible bacteria may become resistant by acquisition of DNA encoding resistance traits

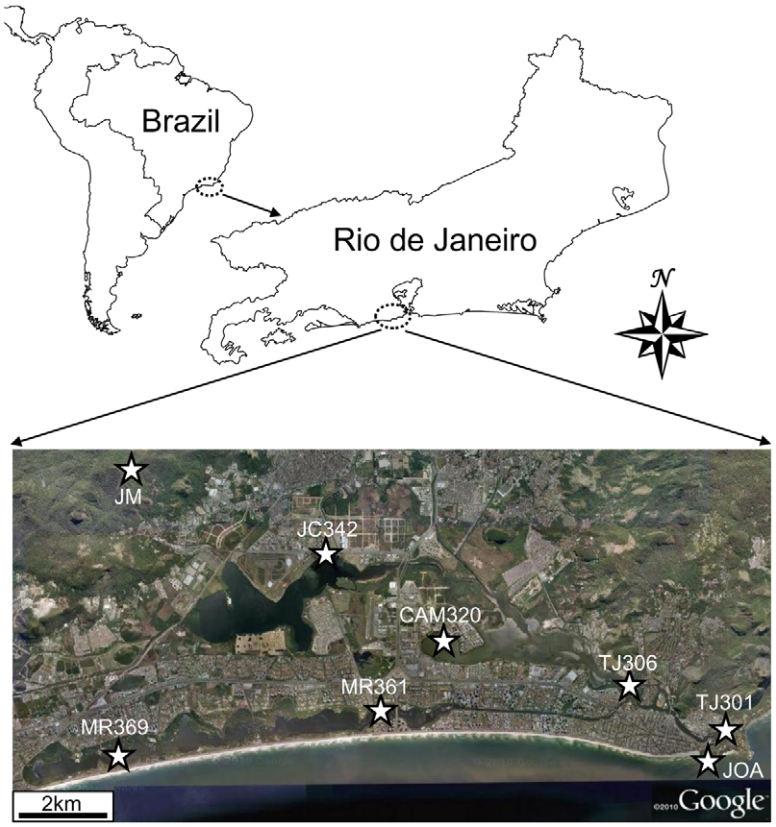


Figure 1. Sampling sites and experimental strategy. Stars (✪) mark sampling points in the lagoon system.
doi:10.1371/journal.pone.0051175.g001

through horizontal gene transfer. This process represents a threat to public health since opportunistic pathogens may acquire drug resistance through this mechanism [12].

Brazil holds around 15% of the Earth's freshwater reserve, distributed across more than 20,000 km^2 of marshlands, rivers, lakes and estuaries. Water pollution occurs mainly in the most industrialized regions in the south and southeast that harbor nearly 60% of the Brazilian population [13]. Risks and consequences associated with microbial transmission in water bodies have been a concern in Brazilian public health issues since 1886 when Oswaldo Cruz earned his medical degree with a thesis addressing the transmission of waterborne microbes. Cruz demonstrated the occurrence of microbes in water from various sources based on physiological and chemical parameters and explored the general prevention of waterborne infections [14].

Application of molecular methods has stimulated the interest in direct monitoring opportunistic and specific pathogens in surface waters [15]. One of the main procedures to evaluate microbial diversity, including pathogen occurrence, is the sequence analysis of environmental genes [16]. This approach can detect uncultured bacteria as well as conventional fecal indicators in water bodies, including *E. coli*, *Salmonella spp*. [17], *Shigella spp*. [18], *Campylobacter spp*. [19], *Legionellae* [20], and *Vibrio vulnificus* [21] among other known pathogens.

The aim of this study was to investigate how bacterial community diversity in a tropical coastal urban lagoon system is related to a pollution and salinity gradient going from terrestrial aquatic habitats up to the coastal Atlantic Ocean. A preserved freshwater environment from the Atlantic rain forest (JM) was used as a pristine control. The lagoon system, comprised of four major lakes (Jacarepaguá, Camorim, Tijuca and Marapendi), is located within the metropolitan area of Rio de Janeiro and covers approximately 280 Km^2. The watershed of Jacarepaguá lagoon system is composed of several rivers that flow to lakes connected to the sea through Joá channel in Barra da Tijuca.

Table 1. Physico-chemical data from sampling stations.[1]

Sites	pH	Conductivity *	Turbidity **	O.D. ***	Salt (%)	Temp (°C)
JM	7.9	0.112	0	5.80	0.00	20.0
JC 342	7.1	4.8	139	5.52	0.25	24.3
CM 320	7.1	6.74	140	6.57	0.36	24.3
MR 369	7.1	9.88	74	4.97	0.55	24.2
TJ 306	7.1	17.8	45	5.50	1.05	24.0
MR 361	7.9	21.7	36	6.17	1.31	24.3
TJ 303	7.6	33.8	22	6.30	2.13	23.8
JOÁ	7.9	50.7	1	5.50	3.34	23.5

MiliSiemens* – mS/cm; ** Nefelométric Turbidity Unity – NTU; * mg/L.
[1]Additional information on physico-chemical parameters of the sampling sites can be found at INEA (http://www.inea.rj.gov.br).
doi:10.1371/journal.pone.0051175.t001

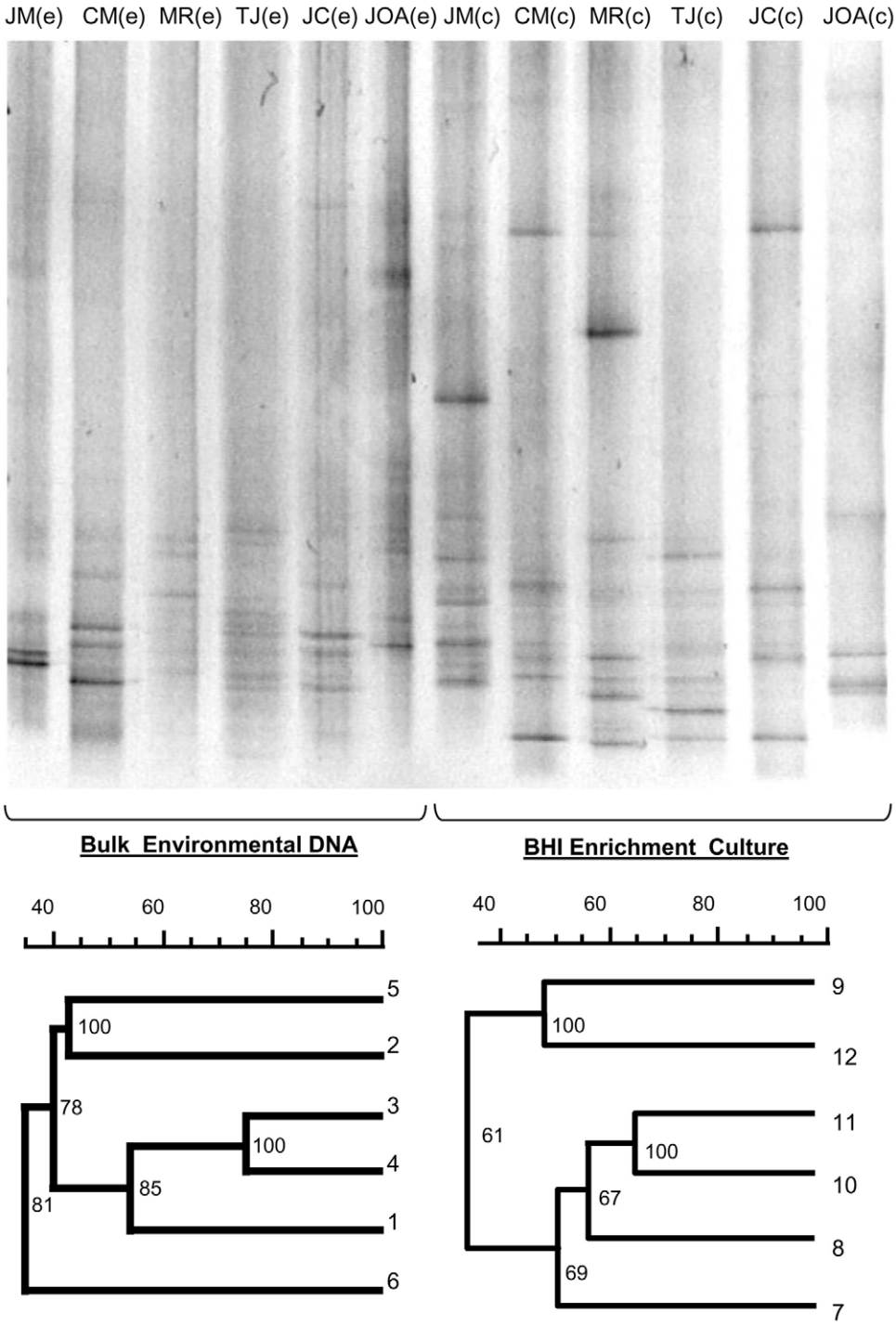


Figure 2. DGGE analysis of environmental and cultured samples. Dendogram generated using Bionumeric software based on images of DGGE band pattern profiles. Each sample is identified as environmental (e) or cultured (c).
doi:10.1371/journal.pone.0051175.g002

Materials and Methods

Ethics statement

An ethics statement is not required for this work. No specific permits were required for the described field studies. The location is not privately-owned or protected in any way and did not involve endangered or protected species.

Site description, experimental design and sampling

The coastal urban watershed of Jacarepaguá, located south of Rio de Janeiro (between latitudes 22°55′S and 23°03′S and longitudes 43°30′W and 43°18′W) is formed by rivers headwaters on the slopes of Tijuca and Pedra Branca massifs, with approximately 280 km^2 of drainage area according to SMAC (Secretaria Municipal de Meio Ambiente). This basin is formed by Tijuca (4.34 km^2), Camorim (0.80 km^2), Jacarepaguá (4.07 km^2)

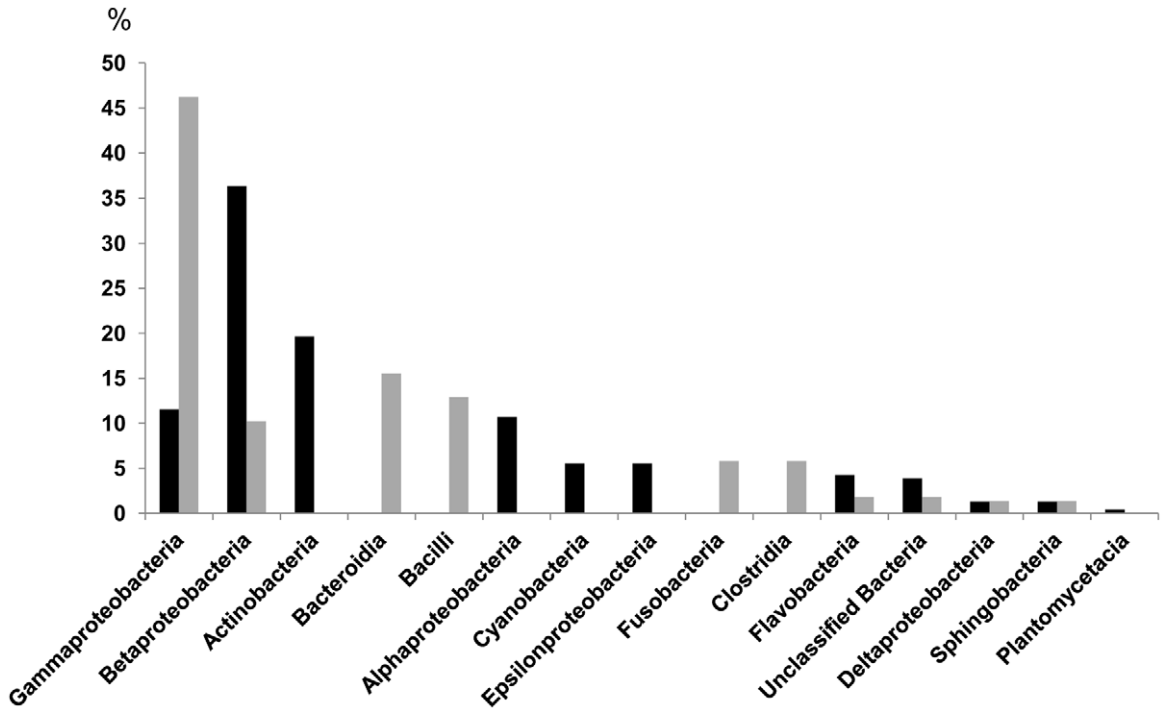


Figure 3. Bacterial taxonomic classes in environmental and cultured libraries. Sequences obtained from 16S rRNA gene libraries retrieved from environmental samples (black) and from enrichment cultures (grey) taxonomic assignment was performed through the RDP Classifier tool.
doi:10.1371/journal.pone.0051175.g003

and Marapendi (3.33 km^2) lakes that are connected to the Atlantic Ocean by Joá channel forming a tide influenced lagoon system. We selected eight sampling sites named: JM, JC342, CM320, MR361, MR369, TJ301, TJ306 and JOA (Figure 1). Samples at each station were obtained on April 16, 2009. JM station is located in a preserved site of the Atlantic rain forest (22°56′85′′S/043°24′6′′W) in a dam of a small pristine river that supplies freshwater to nearby communities. JC342 station (22°58′10′′S/43°22′99′′W) is located near the margin of Jacarepaguá lake close to Pavuna river. CM320 station (22°34′04′′S/043°55′32′′W) is in Camorim lake below the Ayrton Senna bridge. MR361 station (23°01′3′′S/43°25′26′′W) is in Marapendi lagoon at Chico Mendes park. MR369 station (23°00′17′′S/43°21′85′′W) is also in Marapendi lake near a biological reserve. TJ301 station (23°00′33′′S/43°17′62′′W) is in Tijuca lake near the connection to the sea. TJ306 station (23°00′59′′S/43°18′56′′W) is also in Tijuca lake near the junction between Camorin and Marapendi channels. JOA station (23°00′56′′S/43°17′60′′W) is in Joatinga channel under the Joá bridge. Samples of 5.8 L were collected in sterile polypropylene bottles at 1 m deep.

Abiotic parameters

Water samples collected at the eight sites were analyzed for temperature, pH, conductivity, dissolved oxygen, turbidity and salinity at the time of sample collection using Water Quality Checker U-10 (HORIBA). Measurements of total phosphorus, orthophosphate, ammonia, nitrite and nitrate were provided by Instituto Estadual do Ambiente (INEA).

Bacterial isolation and enrichment

Isolation of bacterial strains was performed with inoculants retrieved from the eight sampling sites (Figure 1) using blood agar media (DIFCO) supplemented with 5% sheep blood and four types of commercially available selective culture media: Brain Heart Infusion (BHI) Broth and Agar (MERCK), Cetrimide Agar (CET) (DIFCO), MacConkey Agar (MAC) (MERCK) and Manitol Salt Agar (MSA) (MERCK). Media were prepared according to manufacturer's instructions and autoclaved for 20 min at 121°C. One milliliter of each sample was inoculated in 10 ml of BHI liquid media and incubated at 37°C. After 24 h, cultures were streaked onto BHI, CET, MAC, and MSA agar plates and then incubated at 37°C for 24 to 48 h. Representative morphotypes (twenty-two strains) were selected to perform taxonomic characterization by 16S rRNA gene sequencing. Pure cultures were preserved in glycerol 20% at −70°C.

Water samples were inoculated into Brain Heart Infusion (BHI), a rich culture medium, to promote growth of aerobic and facultative anaerobic bacteria. A one ml aliquot from each of the eight sampling sites (JM, JC342, CM320, MR361, MR369, TJ301, TJ306, and JOA – Figure 1), was inoculated in 10 ml of BHI and incubated at 37°C. After 24 hours, cultures were centrifuged and genomic DNA was extracted from microbial pellets using Qiagen DNeasy Blood & Tissue Kit following manufacturer's recommendations [22].

Antimicrobial susceptibility testing

Determination of antibiotic resistance was performed by a disc diffusion method according to Clinical and Laboratory Standards Institute [23] interpretive criteria recommendations. Bacterial suspensions were adjusted to 0.5 McFarland standards and inoculated into Mueller-Hinton agar medium. Twenty antimicrobial discs were used as follows: ticarcillin/clavulanic acid 75/10 μg, piperacillin/tazobactam 100/10 μg, cefepime 30 μg, ceftazidime 30 μg, aztreonam 30 μg, meropenem 10 μg, imipenem 10 μg, ciprofloxacin 5 μg, gentamicin 10 μg, polymyxin B 300 IU, tobramycin 10 μg, norfloxacin 10 μg, tetracycline 30 μg, ampicil-

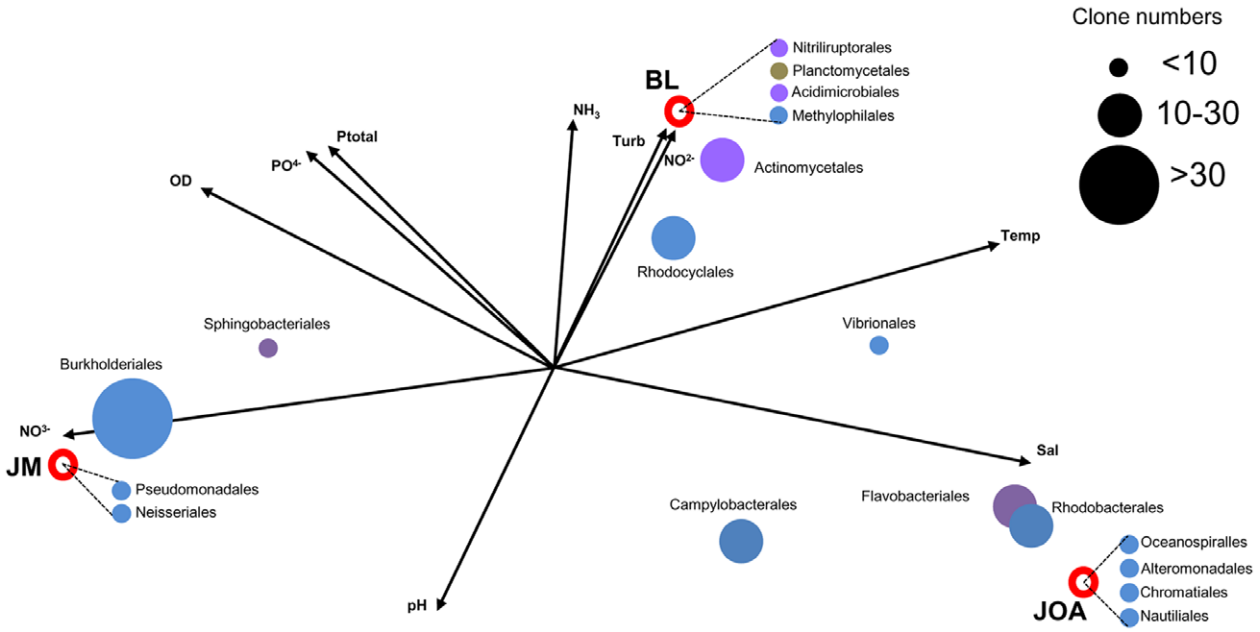


Figure 4. Environmental distribution of bacterial orders. Canonical correspondence analysis of sampling sites libraries from JM(e), BL(e) and JOA(e). Environmental parameters included in this analysis are: total phosphorus (Ptotal), ammonia (NH_3), turbidity (Turb), nitrite (NO_2^-), temperature (Temp), salinity (Sal), pH, nitrate (NO_3^-), dissolved oxygen (OD) and orthophosphate (PO_4^-).
doi:10.1371/journal.pone.0051175.g004

lin 10 μg, cotrimoxazole 25 μg, sulfonamides 300 μg, doxycycline 30 μg, clarithromycin 15 μg, chloramphenicol 30 μg, ampicillin/sulbactam 10/10 μg.

DNA extraction and 16S rRNA gene library construction

Water samples (5.8 L) were filtered through 0.2 μm Sterivex filters (Millipore) after filtration through a 3.0 μm filter to separate

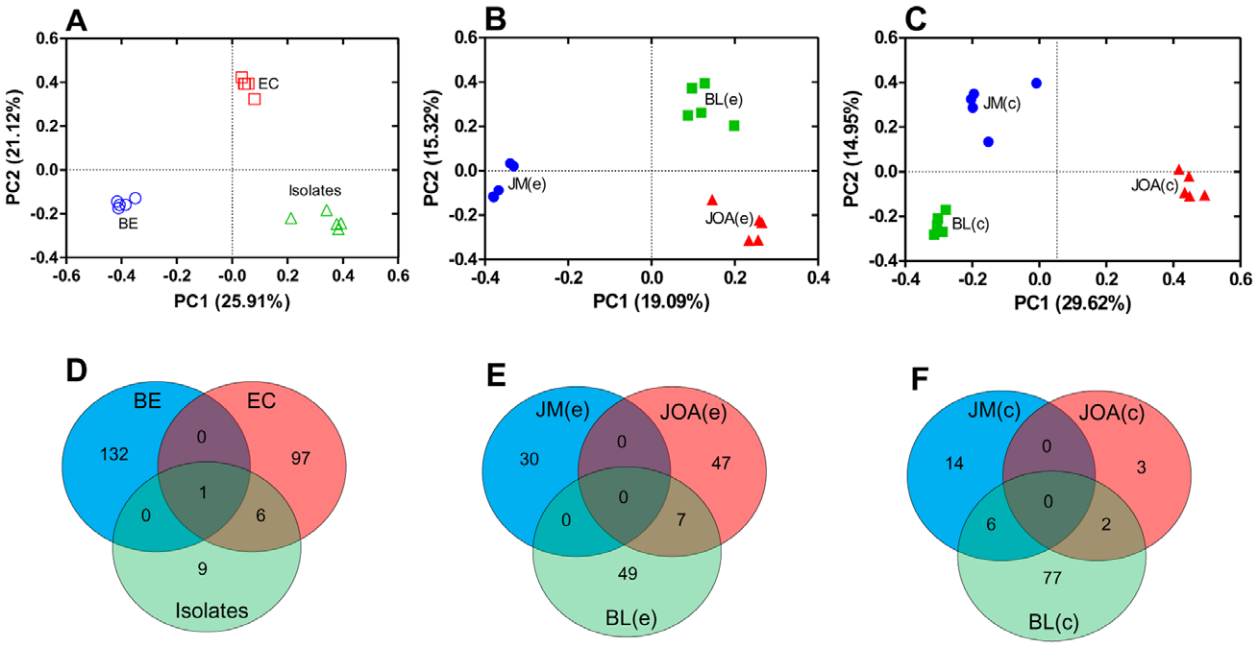


Figure 5. Match and similarities between bacterial communities. Principal coordinates plots (PCoA) were generated using the pairwise unweighted UniFrac distances. (**A**) Comparison between libraries from bulk-environmental DNA (BE), enrichment Cultures (EC) and isolates. (**B**) Comparison between bulk-environmental DNA libraries (e) of marine (JOA), brackish (BL) and freshwater (JM) sampling points, and (**C**) Comparison between enrichment culture libraries (c) of marine (JOA), brackish (BL) and freshwater (JM) sampling points. Venn diagrams (**D**) from libraries of bulk-environmental DNA (BE), enrichment cultures (EC) and isolates. (**E**) Venn diagrams from bulk-environmental DNA libraries (e) of marine (JOA), brackish (BL) and freshwater (JM) sampling points, and (**F**) Venn diagrams from enrichment culture libraries (c) of marine (JOA), brackish (BL) and freshwater (JM) sampling points.
doi:10.1371/journal.pone.0051175.g005

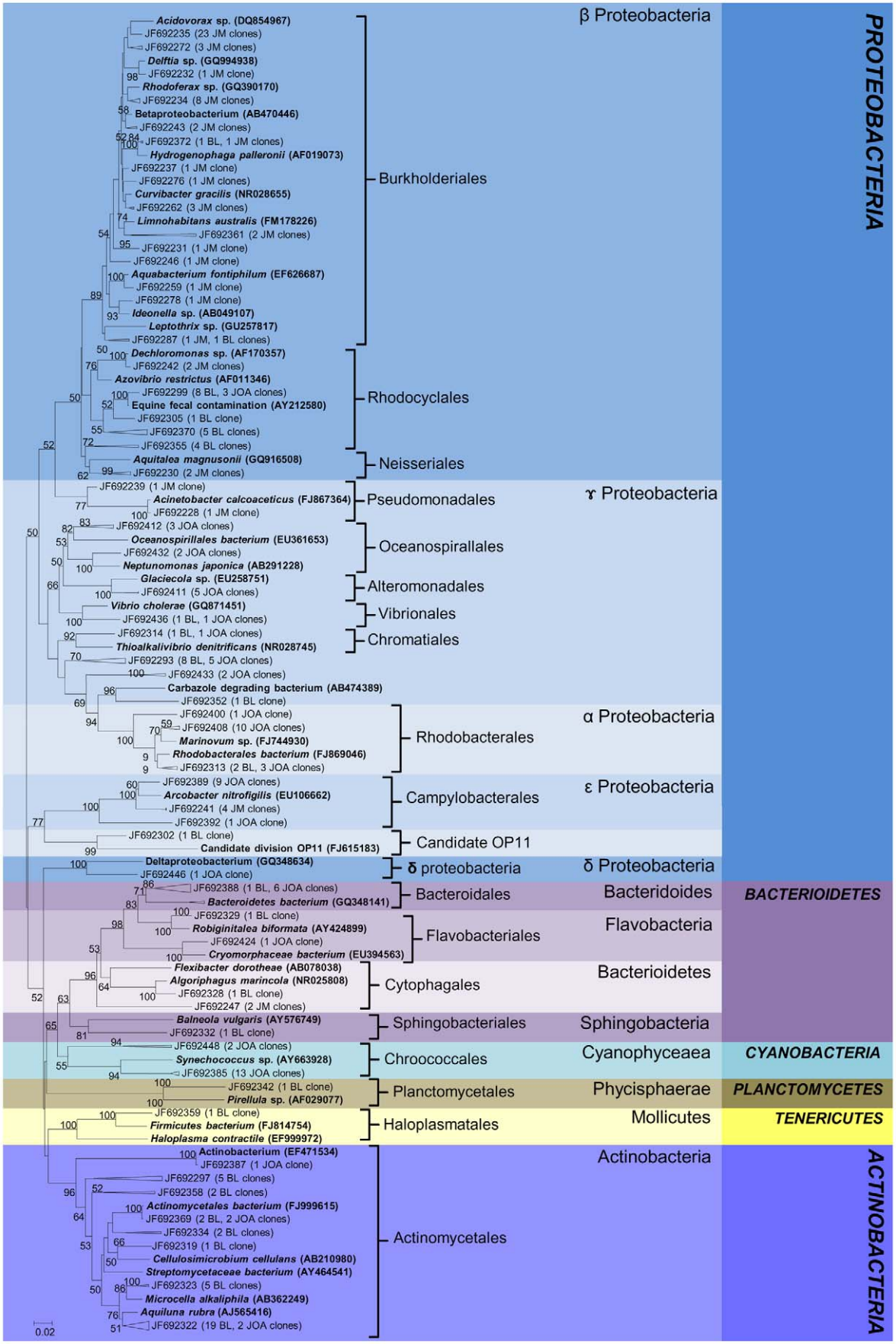

PROTEOBACTERIA
β Proteobacteria
Acidovorax sp. (DQ854967)
JF692235 (23 JM clones)
JF692272 (3 JM clones)
Delftia sp. (GQ994938)
JF692232 (1 JM clone)
Rhodoferax sp. (GQ390170)
JF692234 (8 JM clones)
Betaproteobacterium (AB470446)
JF692243 (2 JM clones)
JF692372 (1 BL, 1 JM clones)
Hydrogenophaga palleronii (AF019073)
JF692237 (1 JM clone)
JF692276 (1 JM clones)
Curvibacter gracilis (NR028655)
JF692262 (3 JM clones)
Limnohabitans australis (FM178226)
JF692361 (2 JM clones)
JF692231 (1 JM clone)
JF692246 (1 JM clone)
Aquabacterium fontiphilum (EF626687)
JF692259 (1 JM clone)
JF692278 (1 JM clone)
Ideonella sp. (AB049107)
Leptothrix sp. (GU257817)
JF692287 (1 JM, 1 BL clones)
Burkholderiales
Dechloromonas sp. (AF170357)
JF692242 (2 JM clones)
Azovibrio restrictus (AF011346)
JF692299 (8 BL, 3 JOA clones)
Equine fecal contamination (AY212580)
JF692305 (1 BL clone)
JF692370 (5 BL clones)
JF692355 (4 BL clones)
Rhodocyclales
Aquitalea magnusonii (GQ916508)
JF692230 (2 JM clones)
Neisseriales
γ Proteobacteria
JF692239 (1 JM clone)
Acinetobacter calcoaceticus (FJ867364)
JF692228 (1 JM clone)
Pseudomonadales
JF692412 (3 JOA clones)
Oceanospirillales bacterium (EU361653)
JF692432 (2 JOA clones)
Neptunomonas japonica (AB291228)
Oceanospirillales
Glaciecola sp. (EU258751)
JF692411 (5 JOA clones)
Alteromonadales
Vibrio cholerae (GQ871451)
JF692436 (1 BL, 1 JOA clones)
Vibrionales
JF692314 (1 BL, 1 JOA clones)
Thioalkalivibrio denitrificans (NR028745)
JF692293 (8 BL, 5 JOA clones)
Chromatiales
JF692433 (2 JOA clones)
Carbazole degrading bacterium (AB474389)
JF692352 (1 BL clone)
α Proteobacteria
JF692400 (1 JOA clone)
JF692408 (10 JOA clones)
Marinovum sp. (FJ744930)
Rhodobacterales bacterium (FJ869046)
JF692313 (2 BL, 3 JOA clones)
Rhodobacterales
ε Proteobacteria
JF692389 (9 JOA clones)
Arcobacter nitrofigilis (EU106662)
JF692241 (4 JM clones)
JF692392 (1 JOA clone)
Campylobacterales
JF692302 (1 BL clone)
Candidate division OP11 (FJ615183)
Candidate OP11
δ Proteobacteria
Deltaproteobacterium (GQ348634)
JF692446 (1 JOA clone)
δ proteobacteria
BACTERIOIDETES
Bacteridoides
JF692388 (1 BL, 6 JOA clones)
Bacteroidetes bacterium (GQ348141)
Bacteroidales
Flavobacteria
JF692329 (1 BL clone)
Robiginitalea biformata (AY424899)
JF692424 (1 JOA clone)
Cryomorphaceae bacterium (EU394563)
Flavobacteriales
Bacterioidetes
Flexibacter dorotheae (AB078038)
Algoriphagus marincola (NR025808)
JF692328 (1 BL clone)
JF692247 (2 JM clones)
Cytophagales
Sphingobacteria
Balneola vulgaris (AY576749)
JF692332 (1 BL clone)
Sphingobacteriales
CYANOBACTERIA
Cyanophyceaea
JF692448 (2 JOA clones)
Synechococcus sp. (AY663928)
JF692385 (13 JOA clones)
Chroococcales
PLANCTOMYCETES
Phycisphaerae
JF692342 (1 BL clone)
Pirellula sp. (AF029077)
Planctomycetales
TENERICUTES
Mollicutes
JF692359 (1 BL clone)
Firmicutes bacterium (FJ814754)
Haloplasma contractile (EF999972)
Haloplasmatales
ACTINOBACTERIA
Actinobacteria
Actinobacterium (EF471534)
JF692387 (1 JOA clone)
JF692297 (5 BL clones)
JF692358 (2 BL clones)
Actinomycetales bacterium (FJ999615)
JF692369 (2 BL, 2 JOA clones)
JF692334 (2 BL clones)
JF692319 (1 BL clone)
Cellulosimicrobium cellulans (AB210980)
Streptomycetaceae bacterium (AY464541)
JF692323 (5 BL clones)
Microcella alkaliphila (AB362249)
Aquiluna rubra (AJ565416)
JF692322 (19 BL, 2 JOA clones)
Actinomycetales
0.02

Figure 6. Phylogenetic tree of bacterial clones from environmental bulk DNA. Reference sequences from GenBank are shown in bold. OTUs were defined by using a distance level of 3% by the furthest neighbor algorithm in MOTHUR. Access number from each OTU is displayed. Tree topology is based on neighbor joining and bootstrap analysis was performed with 1000 replications. Bootstrap value <50 are not shown.
doi:10.1371/journal.pone.0051175.g006

free-living microbes from larger organisms and particles. Total nucleic acids were isolated by cell lysis with proteinase K and SDS, followed by phenol-chloroform extraction as previously described [24]. DNA integrity was checked on a 1% (w/v) agarose gel. PCR was performed in 50 µl reaction mixtures (2.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates, 1 ng.µl^{-1} of each primer, 2.5 U of High Fidelity Platinum *Taq* DNA polymerase (Invitrigen), 1× PCR buffer and 200 ng of each environmental DNA sample, with the universal bacterial primers 27BF (5′-AGAGTTTGATCCTGGCTCAG-3′) [25] and 907RAB (5′-TTTGAGTTTMCTTAACTGCC-3′) [26]. PCR amplification was carried out with a 5 min denaturing step at 94°C; followed by 30 cycles at 94°C for 90 seconds, 50°C for 90 seconds, and 72°C for 2 min. The final step was an extension at 72°C for 5 min. PCR products were concentrated and purified with a GFx PCR DNA and Gel Band Purification Kit (GE Healthcare) after electrophoresis on a 1% (w/v) agarose gel. PCR products were cloned into the pGEM-T cloning vector (Promega) and used to transform competent *E. coli* DH10B cells. Positive colonies were picked and frozen in glycerol 20% at −70°C. While three environmental (e) 16S rRNA gene libraries were constructed from JM(e) (pristine freshwater), BL(e) (DNA pooled from six lake samples) and JOA(e) (coastal marine sample), eight BHI enrichment culture (c) libraries were constructed from JM(c), JC342(c), CM320(c), MR361(c), MR369(c), TJ301(c), TJ306(c) and JOA(c).

DGGE analysis

Bacterial community diversity was analyzed by denaturing gradient gel electrophoresis (DGGE). The 16S rRNA gene fragments were PCR amplified using the specific primers 968BfC (5′-CGCCCGGGGCGCGCC CCGGGCGGGCGGA ACGCGAAGAACCTTAC-3′) and 1401Br (5′-CGGTGTG TACAAGGCCCGGGAACG-3′) [27]. Amplicons (50 µl) from each sample were applied to the DCode DGGE system (BioRad) and ran at 60 V and 60°C for 18 h in 1× TAE buffer. The 6% (w/v) polyacrylamide gels were prepared with a denaturing gradient ranging from 45 to 60%. After electrophoresis, gels were stained with SYBR green I (Molecular Probes) for 1 h and then scanned using the Image Quant 300 (GE Healthcare) gel documentation imager.

Sequence analysis and identification

Approximately 96 clones from each library were selected for sequence analysis. Plasmid DNA from each clone (400 ng) was prepared and sequences were obtained by cycle sequencing with the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and analyzed in an Applied Biosystems ABI Prism 3730 automated DNA sequencer. Chromatograms were converted to Fasta format using Phred software and sequences with less than 300 bp were removed. A total of 494 valid sequences with Phred score ≥20 were compared with sequences from the Ribosomal Database Project II [28]. Sequences were also analyzed by BLAST searches in GenBank database and were aligned with representative bacterial sequences obtained from public databases using ClustalX software [29]. Partial 16S rRNA gene sequences generated in this study were deposited in GenBank under accession numbers JF692227-JF692723.

Canonical Correspondence Analysis

To correlate bacterial diversity with environmental physical and chemical parameters of our sampling sites we performed a Canonical Correspondence Analysis (CCA) carried out through the software MVSP (*MultiVariate Statistical Package 3.1*, Kovach Computing Services). Partial 16S rDNA sequences retrieved from the eight sampling sites were distributed in 18 orders according to their taxonomic assignment performed through the RDP classifier tool. For practical purposes, sequences from six sampling sites located in the lagoon system (JC342, CM320, MR361, MR369, TJ301, and TJ306) were grouped as a single dataset named BL.

Biodiversity and phylogenetic analyses

Chimeric sequences were removed by MOTHUR software [30]. Sequences were clustered as operational taxonomic units (OTUs) at 97% similarity. OTU diversity was examined using rarefaction analysis by the same software. Venn diagrams were also generated using MOTHUR. Phylogenetic trees were constructed from libraries using reference sequences obtained at GenBank by neighbor-joining algorithm, based on distances calculated by the Kimura-2 method. Phylogenetic analysis was performed with MEGA4 software [31] and bootstrap analysis was carried out with 1000 replications. Tree topology and hit distribution were uploaded to the UniFrac computational platform [32]. UniFrac analysis is a beta diversity metric that quantifies sequence similarities based on phylogenetic relatedness. In order to visualize the distribution of bacterial communities we used UniFrac to perform PCoA highlighted by significance. Libraries were randomly sub-sampled to test result consistency.

Results

Water chemistry and experimental design

Abiotic parameters of water samples collected at eight stations (Figure 1) were analyzed. Jacarepaguá and Camorin lakes had high levels of turbidity and low salinity, while low turbidity and high salinity were observed in Marapendi and Tijuca lakes (Table 1). To characterize bacterial communities present in these habitats, culture dependent and independent 16S rRNA gene libraries were constructed.

DGGE analysis of bacterial communities

DGGE profiles retrieved from environmental DNA (BE) and enrichment cultures (EC) were markedly distinct, as well as within the six sites (JM, CM, MR, TJ, JC, JOA) (Figure 2). Relationships among communities resulted in a total of 12 different profiles. Environmental DNA banding patterns produced three clusters. The first included JC and CM lakes grouped at 40% similarity with the second cluster which contained three samples, two from the brackish water lakes MR and TJ, and the other from the freshwater site JM. The most distantly related community is from sea water (JOA) sharing 32% similarity with clusters 1 and 2. Profiles from the enrichment samples resulted in two main clusters related at 35% similarity. The first contained high saline stations MR and JOA (48%) while the second encompassed brackish (JC, TJ, CM) and freshwater (JM) lake samples.

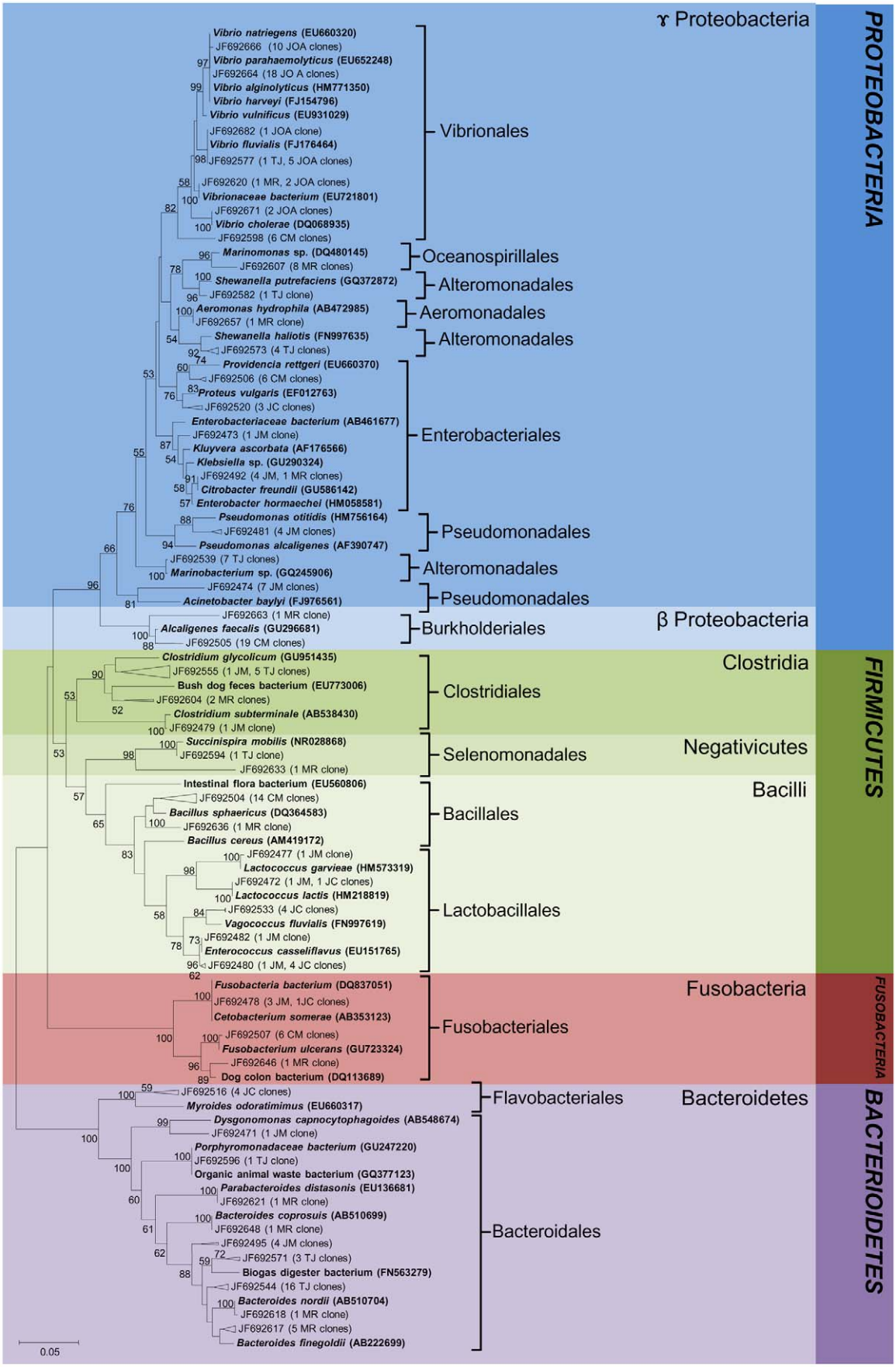

PROTEOBACTERIA
γ Proteobacteria
Vibrio natriegens (EU660320)
JF692666 (10 JOA clones)
Vibrio parahaemolyticus (EU652248)
JF692664 (18 JO A clones)
Vibrio alginolyticus (HM771350)
Vibrio harveyi (FJ154796)
Vibrio vulnificus (EU931029)
JF692682 (1 JOA clone)
Vibrio fluvialis (FJ176464)
JF692577 (1 TJ, 5 JOA clones)
JF692620 (1 MR, 2 JOA clones)
Vibrionaceae bacterium (EU721801)
JF692671 (2 JOA clones)
Vibrio cholerae (DQ068935)
JF692598 (6 CM clones)
Vibrionales
Marinomonas sp. (DQ480145)
JF692607 (8 MR clones)
Oceanospirillales
Shewanella putrefaciens (GQ372872)
JF692582 (1 TJ clone)
Alteromonadales
Aeromonas hydrophila (AB472985)
JF692657 (1 MR clone)
Aeromonadales
Shewanella haliotis (FN997635)
JF692573 (4 TJ clones)
Alteromonadales
Providencia rettgeri (EU660370)
JF692506 (6 CM clones)
Proteus vulgaris (EF012763)
JF692520 (3 JC clones)
Enterobacteriaceae bacterium (AB461677)
JF692473 (1 JM clone)
Kluyvera ascorbata (AF176566)
Klebsiella sp. (GU290324)
JF692492 (4 JM, 1 MR clones)
Citrobacter freundii (GU586142)
Enterobacter hormaechei (HM058581)
Enterobacteriales
Pseudomonas otitidis (HM756164)
JF692481 (4 JM clones)
Pseudomonas alcaligenes (AF390747)
Pseudomonadales
JF692539 (7 TJ clones)
Marinobacterium sp. (GQ245906)
Alteromonadales
JF692474 (7 JM clones)
Acinetobacter baylyi (FJ976561)
Pseudomonadales
β Proteobacteria
JF692663 (1 MR clone)
Alcaligenes faecalis (GU296681)
JF692505 (19 CM clones)
Burkholderiales
FIRMICUTES
Clostridia
Clostridium glycolicum (GU951435)
JF692555 (1 JM, 5 TJ clones)
Bush dog feces bacterium (EU773006)
JF692604 (2 MR clones)
Clostridium subterminale (AB538430)
JF692479 (1 JM clone)
Clostridiales
Negativicutes
Succinispira mobilis (NR028868)
JF692594 (1 TJ clone)
JF692633 (1 MR clone)
Selenomonadales
Bacilli
Intestinal flora bacterium (EU560806)
JF692504 (14 CM clones)
Bacillus sphaericus (DQ364583)
JF692636 (1 MR clone)
Bacillus cereus (AM419172)
Bacillales
JF692477 (1 JM clone)
Lactococcus garvieae (HM573319)
JF692472 (1 JM, 1 JC clones)
Lactococcus lactis (HM218819)
JF692533 (4 JC clones)
Vagococcus fluvialis (FN997619)
JF692482 (1 JM clone)
Enterococcus casseliflavus (EU151765)
JF692480 (1 JM, 4 JC clones)
Lactobacillales
FUSOBACTERIA
Fusobacteria
Fusobacteria bacterium (DQ837051)
JF692478 (3 JM, 1JC clones)
Cetobacterium somerae (AB353123)
JF692507 (6 CM clones)
Fusobacterium ulcerans (GU723324)
JF692646 (1 MR clone)
Dog colon bacterium (DQ113689)
Fusobacteriales
BACTERIOIDETES
Bacteroidetes
JF692516 (4 JC clones)
Myroides odoratimimus (EU660317)
Flavobacteriales
Dysgonomonas capnocytophagoides (AB548674)
JF692471 (1 JM clone)
Porphyromonadaceae bacterium (GU247220)
JF692596 (1 TJ clone)
Organic animal waste bacterium (GQ377123)
Parabacteroides distasonis (EU136681)
JF692621 (1 MR clone)
Bacteroides coprosuis (AB510699)
JF692648 (1 MR clone)
JF692495 (4 JM clones)
JF692571 (3 TJ clones)
Biogas digester bacterium (FN563279)
JF692544 (16 TJ clones)
Bacteroides nordii (AB510704)
JF692618 (1 MR clone)
JF692617 (5 MR clones)
Bacteroides finegoldii (AB222699)
Bacteroidales
0.05

Figure 7. Phylogenetic tree of bacterial clones from BHI enriched culture. Reference sequences from GenBank showcased in bold. OTUs were defined by using a distance level of 3% by using the furthest neighbor algorithm in MOTHUR. Access number from each OTU is displayed. Tree topology is based on neighbor joining and bootstrap analysis was performed with 1000 replications. Bootstrap value <50 are not shown.
doi:10.1371/journal.pone.0051175.g007

Taxonomic composition of 16S rRNA libraries

Using the RDP classifier tool, 16S rRNA gene sequences retrieved from our libraries were assigned to distinct taxonomic classes (Figure 3). Fourteen classes were identified: four of them (*Bacteroidia*, *Bacilli*, *Fusobacteria* and *Clostridia*) were exclusively present in enrichment culture (EC) libraries while five classes (*Actinobacteria*, *Alphaproteobacteria*, *Cyanobacteria*, *Epsilonbacteria* and *Planctomycetacia*) occurred only in environmental libraries (BE). *Gammaproteobacteria* was the most abundant class in EC while *Betaproteobacteria* predominated in BE.

The RDP classifier tool detected 18 bacterial orders between environmental sequences which were distributed in three different environments represented in a CCA diagram (Figure 4) as follows: *Actinomycetales*, *Flavobacteriales*, *Rhodobacterales* and *Vibrionales* were distributed between JOA and BL. *Burkholderiales* and *Sphingobacterales* were common to JM and BL. *Campylobacterales* was found in JOA and JM while *Rhodocyclales* was common to all three environments. All other orders were exclusive from one of the three sampling sites. Orders that include halotolerant organisms were detected exclusively in environments with higher levels of salinity (e.g. *Oceanospirillales* in JOA station and *Vibrionales* in JOA and BL). JOA and JM were positioned in extreme opposites of the CCA diagram; BL was positioned between them but closer to JOA suggesting these two sites share more ecological similarities between themselves than with JM.

Bacterial diversity

Nine 16S rRNA gene libraries were analyzed, three environmental and six from enrichment cultures, yielding a total of 494 valid sequences. Environmental DNA obtained from the four brackish water lakes CM(e), MR(e), TJ(e) and JC(e) were pooled to build a single free living bacterial library henceforth named BL(e). Environmental libraries produced 243 sequences obtained from pristine freshwater JM(e), brackish lagoon water BL(e) and coastal marine seawater JOA(e). A total of 229 sequences were obtained from the six enrichment culture libraries JM(c), JC(c), MR(c), TJ(c), CM(c) and JOA(c). The remaining 22 sequences were recovered from Jacarepaguá lagoon bacterial isolates.

Unweighted UniFrac was used to cluster bacterial 16S rRNA gene sequences according to shared similarities in community composition and applied to simultaneously compare three different data sets through principal coordinate analysis (PCoA) (Figure 5). Initially, sequences from environmental libraries JM(e), BL(e) and JOA(e) were combined as a single data set (BE) and compared with sequences from enrichment cultures (EC) and bacterial isolates (Figure 5A). In the scatter plot, bacterial clusters retrieved by the three different methodological approaches were significantly different. Libraries from environmental sequences (BE) and cultured bacteria (EC and isolates) were separated by the first principal component. Next, environmental pristine freshwater JM(e), Jacarepaguá lagoons BL(e) and coastal seawater JOA(e) libraries were analyzed by PCoA (Figure 5B). The first principal

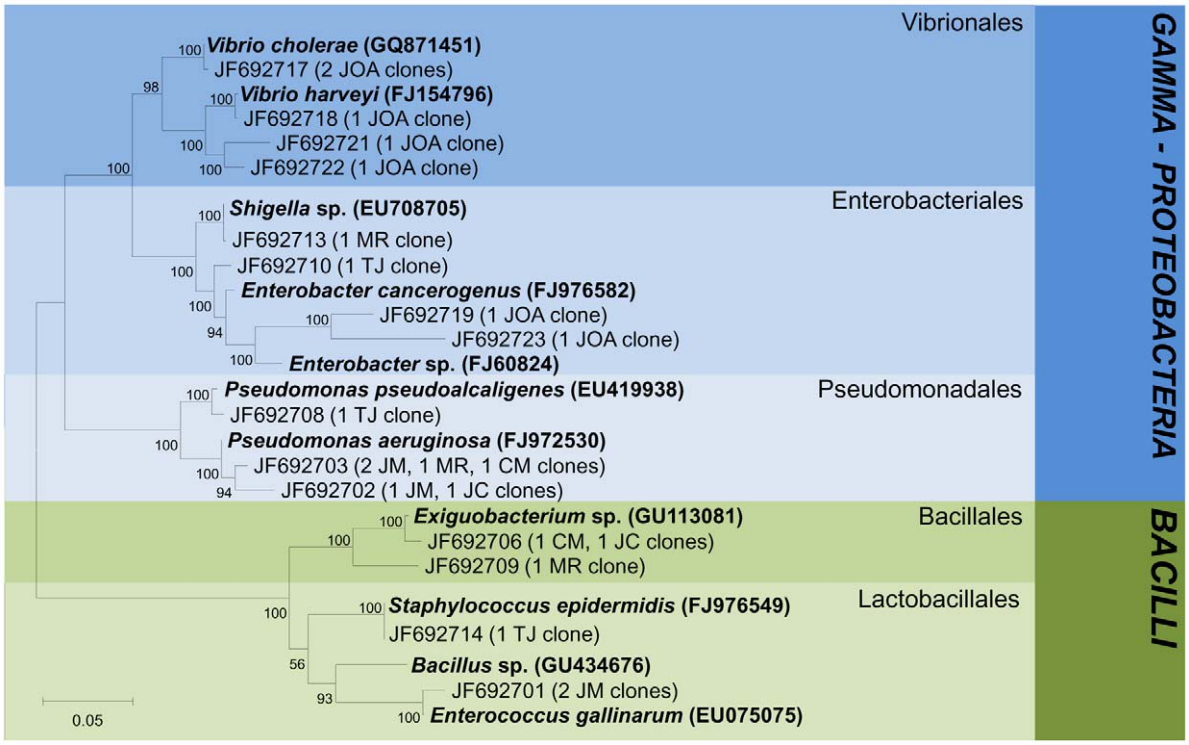


Figure 8. Phylogenetic tree of isolated bacteria. Reference sequences from GenBank are showed in bold. OTUs were defined by using a distance level of 3% by the furthest neighbor algorithm in MOTHUR. Access number from each OTU is displayed. Tree topology is based on neighbor joining and bootstrap analysis was performed with 1000 replications. Bootstrap values <50 are not shown.
doi:10.1371/journal.pone.0051175.g008

Table 2. Antibiotic resistance profile of isolated bacteria.

Isolate	NCBI (BEST HITS) Closest relative and similarity (%)	ACESSION NUMBER	SITE	ANTIBIOTIC Ceftazidime	Aztreonam	Tobramycin
1	[1]*Enterococcus gallinarum (93%)*	EU075075	**JM**	R	R	R
2	[2]*Pseudomonas pseudoalcaligenes (96%)*	EU419938	**TJ303**	S	R	S
3	[2]*Exiguobacterium sp. (96%)*	GU120647	**TJ306**	R	R	S
4	[2]*Exiguobacterium sp.(93%)*	HQ622549	**CM320**	S	R	S
5	[2]*Pseudomonas aeruginosa (95%)*	HM461147	**MR361**	R	R	R
6	[2]*Shigella sp.(96%)*	GU968176	**MR369**	S	S	R
7	[2]*Exiguobacterium sp.(95%)*	AY745848	**JC342**	R	R	S
8	[1]*Vibrio fluvialis (94%)*	FR695475	**JOA**	R	R	R
9	[3]*Vibrio cholerae (96%)*	GQ871451.1	**JOA**	S	R	S
10	[3]*Vibrio harveyi (95%)*	FJ154796.1	**JOA**	S	R	R
11	[4]*Vibrio cholerae (98%)*	CP002555.1	**JOA**	R	R	S

[1]Salt Manitol Agar.
[2]Cetrimide Agar.
[3]Blood Agar.
[4]Mac Conkey Agar.
R, resistant and S, susceptive to antibiotic.
doi:10.1371/journal.pone.0051175.t002

component separated seawater and brackish water libraries from the freshwater sample JM(e). Regarding cultured communities, when JM(c) was compared with BL(c) and JOA(c), principal component 2 separated libraries by the salinity of their original environment (Figure 5C). Community similarity was also assessed through Venn diagrams using OTUs grouped in a similarity level of 97%. First, OTUs from the three methodological approaches (BE, EC and isolates) were compared (Figure 5D). A single OTU is shared between these three datasets, while six were shared between EC and isolates and none were shared between BE and EC or BE and isolates. When analyzing exclusively sequences from environmental samples only JOA(e) and BL(e) shared OTUs (Figure 5E). While sequences from cultures BL(c) shared six OTUs with JM(c) and 2 with JOA(c), none were shared between JM(c) and JOA(c) or between the three datasets (Figure 5F).

Phylogenetic analysis

The identity of each 16S rRNA sequence was determined by BLAST-n searches against the NCBI GenBank database. Bacterial sequences from JM(e) were dominated by *Betaproteobacteria*, especially by members of *Burkholderiales*, *Rhodocyclales* and *Neisseriales* (Figure 6). Two *Cytophagales* clones, four *Arcobacter nitrofigilis* and one clone related to *Acinetobacter* were observed in the freshwater environment. In brackish waters BL(e), the most abundant groups were *Actinomycetales* followed by *Rhodociclales and Rhodobacteriales* that shared the same OTUs with seawater JOA(e). *Vibrionales*, *Chromatiales*, *Bacteroidales*, *Flavobacteriales*, *Sphingobacteriales* and *Planctomycetales* were observed in a smaller proportion. Nine clones related to bacteria originating from equine fecal contamination and a single clone related to candidate division OP11 were also observed in BL(e). In JOA(e), *Cyanobacteria* represented by *Chroococcales* and *Gammaproteobacteria* were dominant groups while only a few sequences were related to *Actinobacteria*, *Deltaproteobacteria* and *Bacteroidetes*. We also observed two clones affiliated to *Vibrio cholerae*, one in seawater and another in the lagoon environment. Sequences obtained from enrichment cultures with different inoculants were distributed into four main bacterial phyla: *Proteobacteria*, *Firmicutes*, *Bacteroidetes and Fusobacteria* (Figure 7). In JM(c), clones related to *Pseudomonadales* were the most abundant, followed by those related to *Bacteroidales*, *Enterobacteriales*, *Lactobacillales*, *Fusobacteriales and Clostridiales*. *Enterobacteriales* was retrieved in brackish water from lakes CM(c), MR(c) and JC(c) while *Pseudomonadales* was present in TJ(c) lake. Clones related to *Clostridiales and Bacteroidales* were shared between MR(c) and TJ(c) lakes while sequences related to *Lactobacillales* were detected in JC(c) lake. Sequences related to *Fusobacteriales* were observed in CM(c) and MR(c) and one clone in JC(c) samples. In MR(c), sequences related to *Oceanospirallales* and *Aeromonadales* were detected. However, clones related to *Burkholderiales* and *Bacillales* were shared with CM(c) and *Selenomonadales* with TJ(c) environments. *Alteromonadales* were observed only in lake TJ(c). The *Vibrionales*, widely represented by several clones, were found in marine water JOA(c) and in lake samples CM(c), TJ(c) and MR(c). We also analyzed phylogenetic relationships of bacteria isolated from freshwater JM(i), brackish water from Jacarepaguá JC(i), TJ(i), MR(i) and CM(i) and marine seawater JOA(i) (Figure 8). Bacterial isolates were composed of two phyla, *Proteobacteria* (72%) and *Firmicutes* (28%) with members divided into *Lactobacillales*, *Enterobacteriales*, *Vibrionales*, *Bacillales* and *Pseudomonadales* orders. Bacteria isolated from JM(i) belonged to *Enterococcus gallinarum* and *Pseudomonas aeruginosa*, which were also isolated from polluted MR(i), CM(i) and JC(i) lakes. Isolates recovered from CM(i), JC(i), MR(i) and TJ(i) samples were related to *Shigella* sp., *Pseudomonas pseudoalcaligenes*, *Staphylococcus epidermidis*, and *Exiguobacteriam* sp.. Bacterial isolates from seawater JOA(i) were affiliated to *Vibrionales* and *Enterobacteriales*. A single OTU, closely related to *Vibrio cholerae*, was shared between the three methodological approaches employed in this study.

Antibiotic susceptibility

From a total of 22 representative morphotypes obtained from different sites at lagoon ecosystems, 11 isolates demonstrated resistance to at least one antibiotic, showcasing five distinct profiles of resistance to β-lactams and aminoglicosides (Table 2). Among twenty antimicrobial test-discs, aztreonam presented the highest resistance profile (91%) as previously demonstrated in the *P. aeruginosa* strains isolated from treated hospital wastewater released in that same aquatic ecosystem [33]. Sequences from the

Jacarepaguá bacterial collection were affiliated with members of *Lactobacillales*, *Enterobacteriales*, *Vibrionales*, *Bacillales* and *Pseudomonadales* orders (Figure 8). Simultaneous resistance to three antibiotics was observed in *Enterococcus gallinarum* from JM, *Pseudomonas aeruginosa* from MR361 and *Vibrio fluvialis* from JOA. In addition, two antibiotic resistant *Vibrio cholerae* isolates from JOA station were detected.

Discussion

Jacarepaguá lagoon ecosystem is a dynamic environment due to mixing of sediments, seawater and continental freshwater impacted by metropolitan pollution. Shifts in physical, chemical and microbiological properties in lagoon and adjacent coastal marine environments can occur in short time periods, driven by marine tides, rainwater runoff and sewage flow, creating an intense selective pressure that influences bacterioplankton community composition in these urban brackish bodies [24,34]. Comparative environmental analysis by CCA (Figure 4) and phylogenetic community comparison by PCoA (Figure 5B) suggest that costal seawater (JOA) and brackish lagoons (BL) develop ecologically similar bacterial communities, probably due to water mixing resulting from tidal variation. Atlantic rain forest freshwater (JM) resulted in lower similarity with the two other sites. Bacterial phylogenetic diversity in these environments is mainly structured by salinity, which explains the distribution of three sampling sites in the CCA diagram: seawater (JOA) and freshwater (JM) are clearly separated and the brackish environment (BL) occupies an intermediate position between them. UniFrac metrics showed that bacterial diversity retrieved from uncultured communities and enrichment cultures were significantly different. PCoA data showed a clear separation between environmental and cultured communities from freshwater (JM), brackish water (BL) and seawater (JOA) samples, corroborating that salinity, rather than temperature and pH, may defines microbial community composition and biogeography as described in other systems [32,35].

It was easy to ascertain through Venn diagrams that a very small number of OTUs were shared between libraries generated from DNA obtained by culture dependent (enrichment and bacterial isolation) and independent methods, suggesting that different bacterial communities are retrieved by each methodology. *Vibrio cholerae* occurrence was detected in seawater and in brackish lagoon environments by all methodological approaches used in this study. Aquatic bacteria might be indigenous to environments or occasionally present in water shedding from human, animal, plant or soil materials [36]. The phylogenetic tree showed significant diversity originating from fecal and pathogenic bacteria residing in polluted lagoon environments (Figure 6).

Fifty percent of the isolates from Jacarepaguá collection were antibiotic resistant bacteria, including species that are known human pathogens such as *Pseudomonas aeruginosa* and *Vibrio cholerae*. Among lagoon isolates, *Enterococcus gallinarum, P. aeruginosa* and *Vibrio fluvialis* showed multi resistance (Table 2). The high level of aztreonam resistance among isolates suggests that environmental strains may act as a reservoir of resistance genes in aquatic ecosystems [37–38]. This achievement may be linked to the occurrence of extended spectrum beta-lactamases (ESBL) and/or ampC-producing organisms, capable of hydrolyzing a large spectrum of cephalosporins, penicillins, and aztreonam [39]. The presence of pathogenic bacteria in polluted lagoons is expected but when these microbes carry genes that confer resistance to conventional drugs these environments pose serious risks to public health [40]. Performing antibiotic susceptibility tests on isolated bacteria is a way to access risks to humans and animals in public environments associated with impacted water bodies. Although numerous previous studies have demonstrated extensive antibiotic resistance in clinical human isolates, much of the immense reservoir of prevalent antibiotic resistance genes present in environmental settings has not been fully described [12,41–42].

Currently, a complementary technique for searching antibiotic resistant genes is metagenomic functional selection, which has demonstrated that the repertoire of resistance genes is much more diverse than previously suggested through the use of culture-dependent methods [43–44]. Alternatively, DNA probes for specific drug resistance genes can be used in metagenomic surveys. Regulatory agencies continue to use traditional indicator counts as a measure of health risk; especially at the local level there is no move toward a sequence based approach to this issue given both costs, technical skill needed and the fact that there is no health assessment available for such sequence analyses. However, massively paralleled deep sequencing approaches are indeed growing in use for community analysis and in the not-so-distant future novel genes and indicators to assess water quality will be revealed.

Acknowledgments

We acknowledge the services of the Johanna Döbereiner Genome Sequencing facilities (IBqM/UFRJ).

We are grateful to Vivian Monteiro for library construction. We also thank Dr Ivano de Filippis for constructive comments and manuscript review.

Author Contributions

Conceived and designed the experiments: MMC RPV OBM. Performed the experiments: GRBS RPV CC JLL. Analyzed the data: AMC MMC OBM FHC LHP RPV. Contributed reagents/materials/analysis tools: MMC AMC RMA OBM. Wrote the paper: GRBS AMC FHC RMA LHP RPV MMC.

References

1. Stewart JR, Gast RJ, Fujioka RS, Solo-Gabriele HM, Meschke JS, et al. (2008) The coastal environment and human health: microbial indicators, pathogens, sentinels and reservoirs. Environ Health 7: 1476–1480.
2. Wu HC, Sercu B, Van De Werfhorst CL, Wong J, DeSantis ZT, et al. (2010) Characterization of Coastal Urban Watershed Bacterial Communities Leads to Alternative Community-Based Indicators. PLoS ONE 5: e11285.
3. Gonzalez-Fernandez D, Garrido-Pérez MC, Nebot-Sanz E, Sales-Márquez D (2010) Fecal Pollution in Coastal Marine Sediments from a Semi-Enclosed Deep Embayment Subjected to Anthropogenic Activities: An Issue to Be Considered in Environmental Quality Management Frameworks Development. Ecohealth. 7: 473–84.
4. Copeland C, Beers B, Thompson M, Pinkerton R, Barrett L, et al. (2009) Faecal contamination of drinking water in a Brazilian shanty town: Importance of household storage and new human faecal marker testing. J Water Health 7: 324–331.
5. Savichtcheva O, Okabe S (2006) Alternative indicators of fecal pollution: Relations with pathogens and conventional indicators, current methodologies for direct pathogen monitoring and future application perspectives. Water Res 40: 2463–2476.
6. Zheng G, Yampara-Iquise H, Jones JE, Carson A (2009) Development of *Faecalibacterium* 16S rRNA gene marker for identification of human faeces. J Appl Microbiol 106: 634–641.
7. Fujioka RS (2001) Monitoring coastal marine waters for spore forming bacteria of fecal and soil origin to determine point from non-point source pollution. Water Sci Technol 44: 181–188.
8. Thompson FL, Iida T, Swings J (2004) Biodiversity of *Vibrios*. Microbiol Mol Biol Rev 68: 403–431.
9. Barba-Brioso C, Fernández-Caliani JC, Miras A, Cornejo J, Galán E (2010) Multi-source water pollution in a highly anthropized wetland system associated with the estuary of Huelva (SW Spain). Mar Pollut Bull 60: 1259–69.

10. Ding C, He J (2010) Effect of antibiotics in the environment on microbial populations. Appl Microbiol Biotechnol 87: 925–941.
11. Bhullar K, Waglechner N, Pawlowski A, Koteva K, Banks ED, et al. (2012) Antibiotic Resistence is Prevalent in an Isolated Cave Microbiome. PLoS ONE 7: e34953.
12. Martínez JL (2008) Antibiotics and antibiotic resistance genes in natural environments. Science 321: 365–367.
13. de Oliveira AJ, Pinhata JM (2008) Antimicrobial resistance and species composition of *Enterococcus* spp. isolated from waters and sands of marine recreational beaches in Southeastern Brazil. Water Res 42: 2242–2250.
14. Cruz OG. A vehiculação microbiana pelas águas [thesis] (1893). Rio de Janeiro: Faculdade de Medicina.
15. Bej AK, Steffan RJ, DiCesare J, Haff L, Atlas RM (1990) Detection of coliform bacteria in water by polymerase chain reaction and gene probes. Appl Environ Microbiol 56: 307–314.
16. Lee DY, Lauder H, Cruwys H, Falletta P, Beaudette LA (2008) Development and application of an oligonucleotide microarray and real-time quantitative PCR for detection of wastewater bacterial pathogens. Sci Total Environ 15: 203–211.
17. Malorny B, Hoofar J, Hugas M, Heuvelink A, Fach P, et al. (2003) Interlaboratory diagnostic accuracy of a *Salmonella* specific PCR-based method. Int J Food Microbiol 31: 241–249.
18. Bej AK, DiCesare JL, Haff L, Atlas RM (1991) Detection of *Escherichia coli* and *Shigella* spp. in water by using the polymerase chain reaction and gene probes for uid. Appl Environ Microbiol. 57(4): 1013–7. Erratum in: Appl Environ Microbiol 57: 2445.
19. Horman A, Rimhanen-Finne R, Maunula L, Von Bonsdorff CH, Torvela N, et al. (2004) *Campylobacter* spp., *Giardia* spp., *Cryptosporidium* spp., noroviruses, and indicator organisms in surface water in southwestern Finland, 2000-2001. Appl Environ Microbiol 70: 87–95.
20. Lee JV, Lai S, Exner M, Lenz J, Gaia V, et al. (2011) An international trial of quantitative PCR for monitoring *Legionella* in artificial water systems. J Appl Microbiol 110: 1032–1044.
21. Chakravorty S, Aladegbami B, Burday M, Levi M, Marras SA, et al. (2010) Rapid universal identification of bacterial pathogens from clinical cultures by using a novel sloppy molecular beacon melting temperature signature technique. J Clin Microbiol 48: 258–267.
22. Orsi RH, Borowsky ML, Lauer P, Young SK, Nusbaum C, et al. (2008) Short-term genome evolution of *Listeria monocytogenes* in a non-controlled environment. BMC Genomics 9: 539–556.
23. CSLI (2010) Performance Standards for Antimicrobial Susceptibility Testing. Twentieth informational Supplement. CSLI document M100-S20. Wayne, PA: Clinical and Laboratory Standards Institute.
24. Vieira RP, Gonzalez AM, Cardoso AM, Oliveira DN, Albano RM, et al. (2008) Relationships between bacterial diversity and environmental variables in a tropical marine environment, Rio de Janeiro. Environ Microbiol 10: 189–199.
25. Lane DJ (1991) 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M, editors. Nucleic Acid Techniques in bacterial systematic. New York: Willey. 115–175.
26. Weisburg WG, Barns SM, Pelletier DA, Lane DJ (1991) 16S ribosomal DNA amplification for phylogenetic study. J Bacteriol 173: 697–703.
27. Watanabe K, Kodama Y, Harayama S (2001) Design and evaluation of PCR primers to amplify bacterial 16S ribosomal DNA fragments used for community fingerprinting. J Microbiol Methods 44: 253–262.
28. Cole JR, Chai B, Marsh TL, Farris RJ, Wang Q, et al. (2003) The Ribosomal Database Project (RDP-II): previewing a new autoaligner that allows regular updates and the new prokaryotic taxonomy. Nucleic Acids Res 31: 442–443.
29. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 24: 4876–4882.
30. Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, et al. (2009) Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol 75: 7537–7541.
31. Kumar S, Nei M, Dudley J, Tamura K (2008) MEGA: a biologist-centric software for evolutionary analysis of DNA and protein sequences. Brief Bioinform 9: 299–306.
32. Lozupone C, Knight R (2007) Global patters in bacterial diversity. Proc Natl Acad Sci USA 104: 11436–11440.
33. Santoro DO, Romão CM, Clementino MM (2012) Decreased aztreonam susceptibility among *Pseudomonas aeruginosa* isolates from hospital effluent treatment system and clinical samples. Int J Environ Health Res. DOI:10.1080/09603123.2012.678000.
34. Crump BC, Armbrust EV, Baross JA (1999) Phylogenetic analysis of particle attached and free-living bacterial communities in the Columbia river, its estuary, and the adjacent coastal ocean. Appl Environ Microbiol 65: 3192–3204.
35. Silveira CB, Vieira RP, Cardoso AM, Paranhos R, Albano RM, et al. (2011) Influence of salinity on bacterioplankton communities from the Brazilian rain forest to the coastal Atlantic Ocean. PLoS ONE 9: e17789.
36. Baquero F, Martínez JL, Cantón R (2008) Antibiotics and antibiotic resistance in water environments. Curr Opin in Biotechnol. 19: 260–265.
37. Canton R (2009) Antibiotic resistance genes from the environment: a perspective through newly identified antibiotic resistance mechanisms in the clinical setting. Clin Microbiol Infect 15: 20–25.
38. Wright GD (2010) Antibiotic resistance in the environment: a link to the clinic? Curr Opin Microbiol 13: 589–594.
39. Rupp ME, Fey PD (2003) Extended spectrum beta-lactamase (ESBL)-producing *Enterobacteriaceae*: considerations for diagnosis, prevention and drug treatment. Drugs 63: 353–365.
40. Aiyegoro O, Adewusi A, Oyedemi S, Akinpelu D, Okoh A (2011) Interactions of antibiotics and methanolic crude extracts of afzelia africana (smith.) against drug resistance bacterial isolates. Int J Mol Sci 12: 4477–4503.
41. Riesenfeld CS, Goodman RM, Handelsman J (2004) Uncultured soil bacteria are a reservoir of new antibiotic resistance genes. Environ Microbiol 6: 981–989.
42. D'Costa VM, McGrann KM, Hughes DW, Wright GD (2006) Sampling the antibiotic resistome. Science 311: 374–377.
43. Sommer MO, Dantas G, Church GM (2009) Functional characterization of the antibiotic resistance reservoir in the human microflora. Science 325:1128–1131.
44. Sommer MO, Church GM, Dantas G (2010) The human microbiome harbors a diverse reservoir of antibiotic resistance genes. Virulence 1: 299–303.