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Detecção e caracterização de virulência e de resistência a drogas antimicrobianas de isolados nosocomiais de *Stenotrophomonas maltophilia*

Porto Alegre

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Dissertação de Mestrado apresentada ao Programa de Pós-Graduação em Biologia Celular e Molecular, da Faculdade de Biociências da Pontifícia Universidade Católica do Rio Grande do Sul.

Orientadora: Profa. Dra. Sílvia Dias de Oliveira

Co-orientador: Prof. Dr. Carlos Alexandre Sanchez Ferreira

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Resumo

Stenotrophomonas maltophilia é um importante patógeno oportunista e emergente, comumente relacionado a infecções nosocomiais e encontrado em diferentes locais, incluindo o ambiente hospitalar. Este microrganismo é reconhecido por apresentar resistência intrínseca a uma gama importante de antimicrobianos, bem como por adquirir resistência através de transferência gênica horizontal, o que reduz as opções efetivas para o tratamento de infecções ocasionadas por este microrganismo. Desta forma, o objetivo deste estudo foi determinar a presença de *S. maltophilia* no ambiente hospitalar e caracterizar a resistência a drogas antimicrobianas dos isolados ambientais, bem como dos isolados clínicos obtidos no mesmo hospital. Para tanto, amostras ambientais foram coletadas na UTI Geral e no andar referente à internação pelo Sistema Único de Saúde (SUS) do Hospital São Lucas, Porto Alegre, Brasil. Além disso, 100 isolados clínicos foram cedidos pelo Laboratório de Patologia Clínica do mesmo hospital. Todas as amostras foram analisadas utilizando um protocolo de detecção específica de *S. maltophilia* através de PCR desenvolvido neste estudo, tendo o gene RNAr 23S como alvo. Posteriormente, foi avaliada a resistência dos isolados frente à ceftazidima, cloranfenicol, levofloxacina, minociclina e trimetoprim/sulfametoxazol (TMP/SMX). A presença de integrons foi verificada em todos os isolados e naqueles com suscetibilidade reduzida a TMP/SMX foi avaliada a presença dos genes *sul*1 e *sul*2, bem como foi determinado o perfil plasmidial. Todos os isolados foram submetidos à detecção do gene *smf*-1. De um total de 936 amostras coletadas no ambiente hospitalar, *S. maltophilia* foi identificada em 28. Foram observadas elevadas taxas de suscetibilidade à minociclina, levofloxacina e cloranfenicol e todos os 19 isolados que apresentaram suscetibilidade reduzida à combinação TMP/SMX carrearam o gene *sul*1, 14 destes apresentaram o integron de classe 1 e nove isolados apresentaram concomitantemente os genes *sul*1 e *sul*2. Todos os isolados que carrearam o gene *sul*2 apresentaram o plasmídeo de 7,3 kb. O gene *smf*-1 foi detectado em 31 isolados de *S. maltophilia*. A presença de *S. maltophilia* nos materiais e equipamentos hospitalares indica a permanência destas bactérias no ambiente hospitalar, podendo constituir risco para infecção de outros pacientes internados. Além disso, os dados obtidos neste estudo em relação à suscetibilidade à TMP/SMX podem sugerir que a resistência a esta combinação de drogas possa estar em ascensão, especialmente porque os determinantes

de resistência a estas drogas podem estar associados a elementos genéticos móveis, o que facilitaria a transferência horizontal e a disseminação destes genes de resistência.

Palavras-chave: *Stenotrophomonas maltophilia*; Infecção hospitalar; Resistência antimicrobiana; Resistência a trimetoprim-sulfametoxazol.

Abstract

Stenotrophomonas maltophilia is an important opportunistic and emerging pathogen commonly related to nosocomial infections and found in different environmental sources, including hospital settings. This microorganism is recognized for presenting intrinsic resistance to a range of important antimicrobials as well as to acquire resistance by horizontal gene transfer, which reduces the effective options for the treatment of infections caused by this organism. Therefore, the aim of this study was to determine the presence of *S. maltophilia* in the nosocomial environment and characterize the resistance of the environmental isolates, as well as clinical isolates obtained in the same hospital. Then, environmental samples were collected in the general ICU and the Unified Health System hospitalization unit of the São Lucas Hospital, Porto Alegre, Brazil. In addition, 100 clinical isolates were sent by the Clinical Pathology Laboratory of the same hospital. All samples were analyzed using a specific protocol for *S. maltophilia* detection by PCR developed in this study targeting 23S rRNA gene. Subsequently, the antimicrobial resistance was evaluated against ceftazidime, chloramphenicol, levofloxacin, minociclin and trimethoprim/sulfamethoxazole (TMP/SMX). The presence of integrons was verified in all isolates and those that presented reduced susceptibility to TMP/SMX was evaluated the presence of *sul*1 and *sul*2 gene, as well as was determined the plasmid profile. All isolates were submitted to detection of *smf*-1 gene. Among the 936 samples collected in the nosocomial environment, *S. maltophilia* was identified in 28. High rates of susceptibility to minocycline, levofloxacin and chloramphenicol were observed, and all of the 19 isolates that presented reduced susceptibility to TMP/SMX carried the *sul*1 gene, 14 of them presented class 1 integron and nine isolates showed simultaneously *sul*1 and *sul*2. All isolates that carried the *sul*2 gene also presented the 7.3 kb plasmid. The *smf*-1 gene was

detected in 31 *S. maltophilia* isolates. The presence of *S. maltophilia* in hospital environment and medical devices indicates the permanence of this microorganism in the nosocomial environment, what can constitute a risk to infection for other hospitalized patients. In addition, the data obtained in this study in relation to TMP/SMX susceptibility can suggest that the resistance to these drugs have the tendency to increase, especially due to resistance determinants to these drugs can be associated to mobile genetic elements, which may facilitate horizontal transfer and the spread of these resistance genes.

Keywords: *Stenotrophomonas maltophilia*; Nosocomial infection; Antimicrobial resistance; Trimethoprim-sulfamethoxazole resistance.

Lista de abreviações

API 20 NE - Analytical profile index for non-enteric Gram-negative rods

BHI - Brain heart infusion

***chit*A -** Chitinase A

CIM - Concentração inibitória mínima

Ct - Threshold cycle

dNTP - desoxinucleosídeos trifosfatados

EDTA - Ácido etilenodiamino tetra-acético

ggps - Glucosylglycerol-phosphate synthase

HSL - Hospital São Lucas

Int - Integrase

LIA - Lysine iron agar

LVX - Levofloxacina

MALDI-TOF - Matrix-assisted laser desorption ionization

MDR - Multidrug Resistance

MIC - Minimal inhibitory concentration

MIN - Minociclina

NaCl - Cloreto de Sódio

PCR - Reação em Cadeia pela Polimerase

PUCRS - Pontifícia Universidade Católica do Rio Grande do Sul

qPCR - Reação em Cadeia pela Polimerase quantitativa

rDNA – DNA ribossômico

RNAr - RNA ribossômico

rRNA - RNA ribossômico

SENTRY - SENTRY antimicrobial surveillance programme

SMF-1 - *Stenotrophomonas maltophilia* type 1 fimbriae

***sul*1 -** dihydropteroate synthase protein 1

***sul*2 -** dihydropteroate synthase protein 2

TMP/SMX - Trimetoprim/Sulfametoxazol

TSI - Triple Sugar Iron

UTI - Unidade de Tratamento Intensivo

Sumário

Capítulo 1

Introdução

Objetivos

1.1 Introdução

Infecções ocasionadas por microrganismos Gram negativos não fermentadores são consideradas de elevada importância clínica e de difícil tratamento devido ao fato destas bactérias comumente apresentarem resistência a múltiplas drogas antimicrobianas (1, 2, 3). A maioria destes microrganismos é capaz de provocar infecções oportunistas associadas a pacientes imunocomprometidos ou em estado crítico, sendo as mais prevalentes: pneumonia, infecções sanguíneas e do trato urinário e infecções em feridas cirúrgicas, enquanto infecções em indivíduos saudáveis são raramente descritas (4, 5, 6). Além disso, estes microrganismos também podem ser identificados no solo e na água, enquanto no ambiente hospitalar, estas bactérias são encontradas principalmente em Unidades de Tratamento Intensivo (UTI), podendo ser isoladas a partir de ventiladores mecânicos, umidificadores, nebulizadores e nas camas dos pacientes, assim como, em equipamentos, acessórios hospitalares e nas mãos dos profissionais (7, 8).

A fibrose cística, uma das doenças genéticas mais frequentes entre indivíduos caucasianos (9), tem sido um fator predisponente para infecções crônicas e recorrentes do trato respiratório ocasionadas por microrganismos Gram negativos não fermentadores emergentes (10). Dentre estes microrganismos destacam-se *Acinetobacter* spp., *Pseudomonas aeruginosa, Stenotrophomonas maltophilia*, bactérias do complexo *Burkholderia cepacia*, algumas espécies dos gêneros *Ralstonia* e *Pandoraea* (7). Muitos destes microrganismos apresentam fenótipos semelhantes, o que faz com que a identificação laboratorial correta destes microrganismos seja extremamente importante para o sucesso do tratamento (11).

O gênero *Stenotrophomonas* compreende bacilos Gram negativos sem arranjo, não fermentadores, aeróbios estritos, móveis, catalase positivos e oxidase negativos

(12). Este gênero é constituído por treze espécies: *S. maltophilia*, *S. rhizophila*, *S. koreensis*, *S. acidaminiphila*, *S. terrae*, *S. humi*, *S. nitritireducens*, *S. panacihumi*, *S. daejeonensis*, *S. detusculanense*, *S. ginsengisoli*, *S. pavanii* e *S. chelatiphaga* (13).

A *S. maltophilia* é um patógeno oportunista e emergente, descrito pela primeira vez em 1958 (Hugh, 1961) (14), sendo encontrada em água, solo, plantas e alimentos (15, 16). Esta bactéria não está presente na microbiota endógena de humanos, porém, pode ser considerada transitória em pacientes hospitalizados comumente colonizando o trato respiratório e digestório (17). Esse microrganismo tem sido isolado em pacientes internados nas UTIs e está associado a infecções nosocomiais, como bacteremias, infecções dos tratos respiratório e urinário, meningites, endocardites e frequentemente presente em pacientes com fibrose cística, bem como em imunocomprometidos (11, 18, 19, 20, 21,, 22). Os fatores de risco para a infecção por *S. maltophilia* incluem procedimentos invasivos utilizados durante hospitalização prolongada, ventilação mecânica, mucosites severas e exposição prévia a antimicrobianos de amplo espectro (23, 24, 25, 26).

Os fatores de virulência associados à *S. maltophilia* estão relacionados com a produção de proteases, lipases e elastases, bem como com a capacidade de aderir fortemente a materiais sintéticos, como cateteres, formando um biofilme, que proporciona proteção contra diferentes agentes antimicrobianos (27, 28, 29, 30). A capacidade de adesão, bem como a produção de biofilme, parece estar relacionada com a produção de fímbrias específicas SMF-1 (28), enquanto a manutenção do biofilme parece depender de *quorum sensing*.

A *S. maltophilia* pode ser identificada através da coloração de Gram e de provas bioquímicas, tais como prova de descarboxiliação d lisina, testes da catalase e da

oxidase, hidrólise da esculina e da gelatina e lise do DNA, assim como a resistência ao imipenem através do crescimento em meio de cultura suplementado com este antimicrobiano (12, 15, 31). Entretanto, ocasionalmente a *S. maltophilia* pode ser identificada erroneamente como *Burkholderia cepacia* (32), necessitando o emprego de métodos mais sofisticados como cromatografia líquida, espectrometria de massa, MALDI-TOF e métodos moleculares (15, 33).

Muitos destes métodos são pouco descritos na literatura para a identificação de microrganismos, e também, apresentam limitações quando utilizados para esta finalidade, tais como custos elevados e problemas na identificação correta dos patógenos (33, 34, 35, 36). Por outro lado, a PCR convencional e a PCR em tempo real são técnicas amplamente utilizadas e descritas como ferramentas importantes para a identificação molecular de *S. maltophilia*. Para tanto, diversos oligonucleotídeos iniciadores tendo como alvo diferentes genes já foram avaliados, tais como: RNAr 16S, RNAr 23S e *chit*A. Entretanto, nenhum dos protocolos desenvolvidos mostrou-se específico para a detecção de *S. maltophilia*, uma vez que detectam também outras espécies do gênero *Stenotrophomonas* e/ou outros bacilos Gram-negativos não fermentadores com características bioquímicas semelhantes (37, 38, 39, 40, 41, 42, 43).

O tratamento de infecções causadas por *S. maltophilia* é difícil e controverso devido à variabilidade genética e fenotípica encontrada entre os microrganismos desta espécie (44). Este microrganismo pode apresentar resistência intrínseca a uma gama importante de antimicrobianos, bem como adquirir resistência através de elementos genéticos móveis como: transposons e plasmídeos, os quais podem carrear determinantes de resistência, muitas vezes inseridos em integrons, que estão relacionados com a diminuição da permeabilidade da membrana, expressão de bombas de efluxo e produção de beta-lactamases (45, 46, 47). Os sistemas de bomba de efluxo

SmeABC e SmeDEF são descritos em *S. maltophilia*, estando entre os principais responsáveis pelo fenótipo de multirresistência. A bomba de efluxo SmeDEF tem sido associada a valores de CIMs aumentados para tetraciclina, cloranfenicol, eritromicina e ofloxacina (48, 49). A hiperexpressão do sistema SmeABC leva à resistência a uma série de antimicrobianos, incluindo aminoglicosídeos, maioria dos beta-lactâmicos e fluoroquinolonas. A resistência aos beta-lactâmicos observada em isolados de *S. maltophilia* que hiperexpressam o sistema SmeABC ocorre devido ao aumento da produção de beta-lactamases e não ao efluxo destes antimicrobianos (46).

A produção constitutiva das beta-lactamases L1 e L2 é considerada o principal mecanismo relacionado à resistência intrínseca aos beta-lactâmicos em *S. maltophilia*. A L1 metalo-beta-lactamase é capaz de hidrolisar as drogas pertencentes às classes de beta-lactâmicos, incluindo penicilinas, cefalosporinas e carbapenêmicos, e não é inibida pelo ácido clavulânico. Enquanto a L2 serina-beta-lactamase é uma cefalosporinase capaz de hidrolisar aztreonam e é inibida parcialmente por outros inibidores de beta-lactamase (50, 51, 52).

A expressão de dois ou mais mecanismos resultam no desenvolvimento de resistência a múltiplas drogas (MDR) e, nesta condição, faz-se necessária à utilização de agentes antimicrobianos em associação (53). A combinação de trimetoprim-sulfametoxazol (TMP-SMX) é a terapia de escolha para o tratamento de infecções ocasionadas por *S. maltophilia* (51, 54). Entretanto, já existem relatos de resistência a estas drogas (55, 56), que pode ser mediada pela presença de integron classe 1 carreando os genes *sul*1 e/ou *sul*2 (57, 58). É importante destacar a carência de informações referentes à resistência de *S. maltophilia* a esta combinação de drogas, considerada a terapia de escolha para o tratamento de infecções ocasionadas por esta bactéria no Brasil. Além disso, autores relatam que os genes *sul*, especialmente o gene

*sul*2, e integrons, podem estar inseridos em plasmídeos, aumentando as possibilidades de transferência dos genes de resistência a outras bactérias (58, 59). Hu e colaboradores (2011) (59) corroboram esta hipótese, uma vez que detectaram a presença do plasmídeo de 7.3 kb em todos os 31 isolados de *S. maltophilia* resistentes a sulfametoxazol analisados. Assim, a determinação do perfil plasmidial dos isolados de *S. maltophilia* pode fornecer um indicativo da presença ou não de genes de resistência às sulfonamidas.

Além disso, dados reportados pelo Programa de Vigilância Antimicrobiana SENTRY, demonstraram a existência de variação da sensibilidade antimicrobiana apresentada por isolados de *S. maltophilia* à TMP/SMX, dependendo da região de procedência. No Canadá e na América Latina, foi descrita uma resistência de 2% ao TMP-SMX, enquanto na Europa foi observada a taxa de 10%. Essa diferença geográfica de resistência sugere a necessidade de um número maior de estudos regionais para a determinação de resistência a esta droga (60). Com o objetivo de contornar a resistência a trimetoprim-sulfametoxazol, têm sido utilizadas associações destes antimicrobianos com ciprofloxacina ou tobramicina, obtendo-se resultados mais satisfatórios quando comparadas ao tratamento somente com TMP-SMX (Zelenitsky e colaboradores (2005) (61) e por Al-Jasser e colaboradores (2006) (56).

Outras drogas antimicrobianas, como minociclina e levofloxacina, estão sendo testadas *in vitro* com o objetivo de avaliar sua atividade contra isolados de *S. maltophilia*. Estudos demonstram que isolados de *S. maltophilia* apresentam elevadas taxas de suscetibilidade quando testados frente a estes antimicrobianos, podendo representar uma alternativa para o tratamento de infecções ocasionadas por esta bactéria (62, 63, 64, 65).

1.2 Objetivos

1.2.1 Objetivo Geral

Este trabalho teve como objetivo a detecção e a determinação da resistência a drogas antimicrobianas e do perfil plasmidial de isolados nosocomiais de *Stenotrophomonas maltophilia*.

1.2.2 Objetivos Específicos

1.2.2.1 Padronizar a detecção de *S. maltophilia* através de PCR convencional e de PCR em tempo real;

1.2.2.2 Isolar e identificar *S. maltophilia* a partir de amostras ambientais da UTI Geral Adulto e do 6° andar referente à internação SUS do Hospital São Lucas da PUCRS através de métodos fenotípicos e genotípicos;

1.2.2.3 Determinar a resistência a drogas antimicrobianas de isolados de *S. maltophilia* oriundos do ambiente hospitalar, bem como de isolados clínicos obtidos no laboratório do mesmo hospital, através da técnica de difusão de discos em agar;

1.2.2.4 Determinar a concentração inibitória mínima para ceftazidima e cloranfenicol em isolados clínicos e ambientais de *S. maltophilia*;

1.2.2.5 Detectar a presença de integrons através da PCR em isolados de *S. maltophilia*;

1.2.2.6 Detectar a presença dos genes *sul*1 e *sul*2 em isolados de *S. maltophilia*;

1.2.2.7 Detectar o gene *smf*-1 em isolados de *S. maltophilia*;

1.2.2.8 Determinar o perfil plasmidial dos isolados de *S. maltophilia* resistentes a trimetoprim-sulfametoxazol.

Capítulo 2

A novel specific PCR method to identify *Stenotrophomonas maltophilia*

Artigo científico submetido ao periódico científico *Memórias do Instituto Oswaldo Cruz*.

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A novel specific PCR method to identify *Stenotrophomonas maltophilia*

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Summary

Stenotrophomonas maltophilia is a multidrug-resistant nosocomial pathogen and its unequivocal identification is not achieved with current methods. So, as the presence of this microorganism on a patient may directly determine the antimicrobial treatment to be performed, conventional PCR and Real-Time PCR assays targeting 23S rRNA were developed to identify specifically *S. maltophilia*. The PCR protocol showed high specificity when tested against other species of *Stenotrophomonas* and non-fermentative Gram-negative bacilli, as well as, against 100 clinical isolates of *S. maltophilia* previously identified by Vitek System.

Key words: *Stenotrophomonas maltophilia* - identification - PCR

Financial support: CAPES, Brazil

Stenotrophomonas maltophilia is an important emerging opportunistic nosocomial pathogen found in different environmental sources, being commonly described as an infecting agent in immunocompromised, oncologic and cystic fibrosis patients (Davies & Rubin 2007, Looney et al. 2009). Treatment of infection caused by *S. maltophilia* is difficult to be performed due to the intrinsic resistance to important antimicrobial agents as well as to mobile genetic elements, such as transposons and plasmids, which are frequently present carrying resistance determinants inserted in integrons (Chang et al. 2004, Liaw et al. 2010, Nicodemo & Paez 2007).

S. maltophilia is usually isolated by selective medium with the addition of imipenem and other antimicrobial agents and identified by biochemical methods (Adjidé et al. 2010, Foster et al. 2008a). However, biochemical tests, including commercial systems such as Vitek-2, API-20NE and Biolog, which are used to identify *S. maltophilia*, sometimes misidentify it as other non fermentative Gram-negative bacilli (Pinot et al. 2011, Zbinden et al. 2007). Moreover, PCR-based protocols for the identification of *S. maltophilia* have also presented limitations related to low specificity due to significant genetic similarity between the *Stenotrophomonas* species and other non-fermentative Gram-negative bacilli (Berg et al. 1999, Riley et al. 2008).

To overcome this problem, we developed a specific PCR protocol for the identification of *S. maltophilia* based on the design of a primer pair that target a fragment of 278 bp of the 23S rRNA gene (F: 5'GCTGGATTGGTTCTAGGAAAACGC3' and R: 5'ACGCAGTCACTCCTTGCG3'). The 23S rRNA gene was chosen due to higher variability of this region among species of the *Stenotrophomonas* genus when compared to the 16S rRNA gene. The PCR mixture was constituted by 100 ng of target DNA extracted according to Rademaker and de Bruijn (Rademaker & de Bruijin 1997),

0.2 mM of each deoxynucleoside triphosphate (dNTP), 2.5 µL of 10X PCR buffer (Invitrogen), 1.0 mM $MgCl_2$, 0.2 U *Taq* DNA polymerase (Invitrogen) and 20 µM of each primer in a total volume of 25 µL. Amplifications were performed in Veriti Thermal Cycler (Applied Biosystems) using an initial step of denaturation at 94ºC for 5 min followed by 30 cycles of denaturation at 94ºC for 45 sec, annealing at 68ºC for 45 sec and extension at 72ºC for 45 sec, with a final extension at 72ºC for 10 min. The amplicons were analyzed by electrophoresis on a 2.0% agarose gel. In addition, the same primer pair was evaluated in a real-time PCR assay using a Platinum SYBR Green qPCR SuperMix- UDG (Invitrogen). Each 20 µL reaction contained 100 ng of DNA sample, 10 µL of SYBR Green qPCR SuperMix reagent (Invitrogen) and 0.2 µL of each primer. The amplifications were performed in Step One Real-Time PCR (Applied Biosystems) using the same annealing temperature and cycles number used in the conventional PCR.

Reference strains of the *Stenotrophomonas* species and other non-fermentative Gram-negative bacilli were used to standardize the protocol: *S. maltophilia* ATCC 13637; *S. maltophilia* LMG 958T; *S. maltophilia* former *Stenotrophomonas africana* LMG 22072 (Coenye et al. 2004); *Stenotrophomonas acidaminiphila* LMG 22073; *Stenotrophomonas nitritireducens* LMG 22074; *Acinetobacter baumannii*; *Burkholderia cepacia*; *Pseudomonas aeruginosa* and *Xanthomonas axonopodis*. Specific amplifications were detected for both PCR methods only for the *S. maltophilia* strains, including the sample previously classified as *S. africana*, and no amplification was observed for all other bacterial strains tested (Fig. 1 and Fig. 2). The detection limits of real-time PCR were determined using threshold cycle (Ct) values obtained from tests performed with *S. maltophilia* ATCC 13637 and *S. maltophilia* former *S. africana* LMG 22072, what present Ct 19.58 and 15.9, respectively. All tests were performed in

duplicate. The value assigned to the standard deviation was 0.08 for both analyses. In order to test the applicability of the protocol, 100 different clinical isolates of *S. maltophilia*, previously identified by Vitek System (bioMérieux, Hazelwood, MO, USA), were evaluated and produced specific amplicons.

One amplicon obtained from *S. maltophilia* ATCC 13637 was randomly chosen and sequenced in an ABI 3130 XL Genetic Analyzer (Applied Biosystems) automated DNA sequencer. The sequence was aligned with *S. maltophilia* 23S rRNA sequences deposited in GenBank (AM743169 and AF273255) through MEGA 5.10 Beta software, presenting 100% identity.

Foster et al. (2008b) and Pinot et al. (2011) have used a different region of the 23S rRNA gene as target, but showed cross-reaction between *Stenotrophomonas* and *Xanthomonas* species, demonstrating low PCR specificity. Pinot et al. (2011), additionally, developed a multiplex PCR targeting *sme*D and *ggp*S to differentiate *S. maltophilia* and *S. rhizophila*, what was accomplished, although did not exclude the cross-reaction with *Xanthomonas* species. As can be seen in Fig. 1, no cross-reaction was observed with the *X. axonopodis* using the protocol described in this study. However, the primer pair was not evaluated against other species of *Xanthomonas* and/or *Stenotrophomonas rhizophila*. Other studies had already described protocols to detect *S. maltophilia* by real-time PCR, but the cross-reaction with *Xanthomonas* and/or other *Stenotrophomonas* species were not be evaluated (Dark et al. 2011, Wellinghausen et al. 2004).

Therefore, the primers designed in this study show an important alternative to detect specifically *S. maltophilia* using rapid molecular methods, which can enable a faster choice for an appropriate antimicrobial protocol in order to obtain success in the treatment of infections caused by this microorganism.

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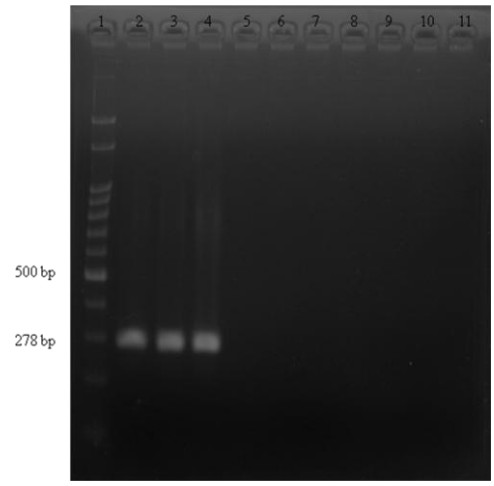

Fig. 1: electrophoresis of amplificons from 23S rRNA gene on 2.0% agarose gel stained with ethidium bromide. Lanes 2 to 10 - *Stenotrophomonas maltophilia* ATCC 13637, *S. maltophilia* LMG 958T, *S. maltophilia* LMG 22072 former *Stenotrophomonas africana*, *Stenotrophomonas acidaminiphila* LMG 22073, *Stenotrophomonas nitrireducens* LMG 22074, *Acinetobacter baumannii*, *Burkholderia cepacia*, *Pseudomonas aeruginosa* and *Xanthomonas axonopodis*; lane 11 - negative control; lane 1 - 100 bp molecular mass marker (Ludwig Biotecnologia).

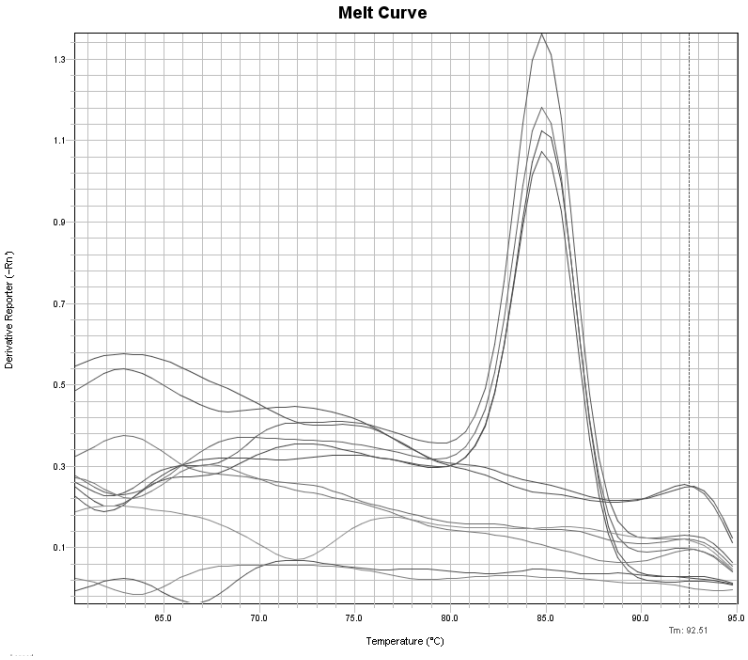

Fig. 2: melting curve obtained by the Real-Time PCR analysis. The PCR demonstrated the same melting point for *Stenotrophomonas maltophilia* ATCC 13637 and *S. maltophilia* LMG 958T performed in duplicate. No amplification product was observed by the other species of *Stenotrophomonas* and non-fermentative Gram-negative bacilli.

Capítulo 3

Detection and characterization of virulence and antimicrobial resistance of *Stenotrophomonas maltophilia* nosocomial isolates

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DETECTION AND CHARACTERIZATION OF VIRULENCE AND ANTIMICROBIAL RESISTANCE OF *Stenotrophomonas maltophilia* NOSOCOMIAL ISOLATES

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SUMMARY

Background: *Stenotrophomonas maltophilia* is an emerging multidrug-resistant nosocomial pathogen responsible to cause several infections in immunocompromised patients that can be found in the hospital environment.

Aim: Evaluate the presence of *S. maltophilia* in the nosocomial environment, determine the antimicrobial susceptibility and characterize the virulence of clinical and environmental isolates obtained from the same hospital.

Methods: 936 samples were collected from nosocomial environment. The *S. maltophilia* identification was performed by PCR targeting 23S rRNA gene and the antimicrobial resistance was characterized. The presence of integrons, *sul*1, *sul*2 and *smf*-1 genes was also evaluated by PCR. The plasmid profile was further analized in all isolates presenting resistance to trimethoprim-sulfamethoxazole.

Findings: *S. maltophilia* isolates were detected in 3% of samples collected from nosocomial environment, and 100 clinical isolates were sent by the laboratory of the same hospital. The majority of the isolates was susceptible to levofloxacin, minocycline and chloramphenicol. High resistance to ceftazidime was detected in both groups of isolates. Among the isolates resistant to TMP/SMX, all presented *sul*1 gene, and 64.3% clinical and 60% environmental isolates carried the *sul*2 gene. Class 1 integron was detected in 10.9% of isolates. All isolates that carried *sul*2 gene presented a 7.3 kb plasmid. The presence of *smf*-1 gene was detected in 31 isolates.

Conclusion: The presence of *S. maltophilia* in the nosocomial environment indicates it as possible reservoir of resistant and pathogenic *S. maltophilia*. In addition, resistance to TMP/SMX was found in *S. maltophilia*, what can constitute great concern if it indicates a tendancy to increase and spread.

Keywords: *Stenotrophomonas maltophilia*; Antimicrobial resistance; Nosocomial infection; *sul* genes; Sulfametoxazole resistance

Introduction

Stenotrophomonas maltophilia is an important opportunistic pathogen commonly found in environment, including hospital settings.[1-4] The ability of this microorganism to survive in the environment under adverse conditions is related to its strong capability of adhesion to synthetic materials, abiotic surfaces and its biofilm production, which can be due to the presence of the virulence gene *smf*-1 that encondes a type-1 fimbriae (SMF-1).[5-8] *S. maltophilia* has been also isolated from patients hospitalized in intensive care units (ICUs), and often related with cystic fibrosis and immunocompromised patients.[9-14]

The treatment of infections caused by *S. maltophilia* is usually problematic to be performed due to its intrinsic resistance to a broad spectrum of antimicrobial agents, including aminoglycosides, fluoroquinolones and the majority of beta-lactams. The antimicrobial resistance of this microorganism is assigned to genetic determinants related with a reduced membrane permeability as well as efflux pumps expression which can often determine a multiresistant phenotype.[15-20] Therefore, it is commonly required the use of antimicrobial agents combination, mainly the association of trimethoprim-sulfamethoxazole (TMP/SMX), which is the first therapeutic option for the treatment of infections caused by *S. maltophilia*. However, isolates harboring *sul*1, *sul*2, or both, have been described as resistant to this combination. The *sul*1 gene is carried as part of the 3' end of class 1 integron and can be located in the chromosome or carried by plasmids,[21,22] while the *sul*2 gene is present primarily on plasmids, characteristic that probably facilitate the horizontal spread of resistance to sulfametoxazole through bacterial populations.[22, 23]

Therefore, the aim of this study was to determine the presence of *S. maltophilia* in the nosocomial environment as well as to characterize the antimicrobial resistance and virulence in clinical and environmental isolates.

Materials and methods

Samples

During November 2010 to September 2011, weekly, 25 environmental and 1 floor samples were collected from different places of nosocomial environment, such as bed, bidet, mechanical ventilator, infusion pump and floor, making a total of 936 samples. All samples were collected in the general adult ICU (n=546) and the non-ICU unit (n=354) at a 603-bed university hospital located in the city of Porto Alegre, Southern Brazil. The samples were collected with a swab immersed in 0.1% saline peptone, while the floor samples (n=36) were collected using a "drag swab" soaked in 0.1% saline peptone. In the same period, the Department of Microbiology of the Clinical Pathology Laboratory of the same hospital sent 100 strains of *S. maltophilia* from clinical specimens previously identified using the Vitek System (bioMérieux, Hazelwood, MO, USA), what were previously confirmed as *S. maltophilia* by PCR using 23S rRNA gene as target (Gallo et al., 2012, submitted).[24] Among the 100 clinical strains, 55 were obtained from tracheal aspirate, 15 blood culture and 14 sputum samples, followed by 4 pleural fluid, 3 oropharynx secretion, 2 urine, 2 ascites fluid, 1 bronchial lavage, 1 wound operative, 1 catheter tip, 1 secretion from foot and 1 biopsy of ulcer.

Isolation and identification of S. maltophilia

The material collected from hospital environment was inoculated in 3 mL of BHI broth (Himedia, India), incubated in agitation at 37°C for 24h, and spread on MacConkey agar (Oxoid, UK). An aliquot of 100 μL of saline peptone used as transport of the floor sample was spread on blood agar and incubated at 37°C for 24h. The colonies with morphology compatible with *S. maltophilia* were submitted to biochemical tests: oxidase (Laborclin, Brazil), triple sugar iron (TSI) (Oxoid, UK), lysine iron agar decarboxylation (LIA) (Merck, Germany) and esculin hydrolysis (Himedia, India).

The isolates presumptivelly identified as *S. maltophilia* through biochemical tests were submitted to a PCR assay. The target DNA was extracted with a guanidine isothiocyanate protocol (Rademaker and de Brujin, 1997)[25] and used as template to amplify a 278 bp fragment of the 23S rRNA gene according to Gallo et al. (2012, submitted).[24] *S. maltophilia* ATCC 13637 was used as reference culture in biochemical and molecular tests.

Antimicrobial susceptibility testing

The antimicrobial resistance pattern was performed according to the guidelines recommended by Clinical and Laboratory Standards Institute (CLSI, 2012)[26] using the disk diffusion technique. The antimicrobials and concentration in micrograms tested were: levofloxacin (LVX), 5; minocycline (MIN) 30; and trimethoprim-sulfamethoxazole (TMP/SMX), 25.

The minimal inhibitory concentration (MIC) to ceftazidime and chloramphenicol was determined by agar dilution method using a commercial Mueller-Hinton agar (Himedia, India) and broth microdilution method. The Mueller-Hinton broth (Himedia,

India) was prepared from the powder and supplemented with Ca^{++} and Mg^{++}, according recommendations of CLSI guidelines[26], ranging from 1 μg/mL to 128 μg/mL. The results were interpreted according to CLSI (2012)[27]. *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as reference cultures for the antibiotic quality control in all antimicrobial resistance tests.

Essential agreement was determined to be when the agar dilution results agreed within ±1 $\log_2$ dilution compared to the reference broth microdilution method. The result was considered discrepant when the dilution difference between results was ±2 $\log_2$. The categorical agreement was established to evaluate if the results were in the same susceptibility category and the errors were classified as: very major error, false-susceptible result by agar dilution; major error, false-resistant result by agar dilution; and minor error, intermediate result by agar dilution method and resistant or susceptible for the broth microdilution test.[28]

Detection of antimicrobial resistance and virulence determinants

The presence of class 1, 2 and 3 integrons was detected by PCR using a degenerate primer pair able to identify genes from integrases *intI*1, *intI*2 and *intI*3.[29] The isolates positive for these integrases were submitted to a specific PCR targeting the *intI*1 gene.[29] The PCR assays targeting different integrases and the *intI*1 gene were performed using the same conditions: 0.2 mM of each deoxynucleoside triphosphate (dNTP), 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, 0.2 U *Taq* DNA polymerase (Invitrogen) and 20 μM of each primer in a total volume of 25 μL. Except for altering the annealing temperature to 57°C for the PCR using a degenerate primer pair and to 55ºC for the *intI*1 gene. Sulfamethoxazole resistance determinants were detected by PCR using primers previously described targeting *sul*1 and *sul*2 genes.[30, 31] The $MgCl_2$ concentrations and the anneling temperature used to detect the *sul* genes were different

to that described by the authors: 2.0 mM $MgCl_2$ and 64ºC to *sul*1 gene, and 1.5 mM $MgCl_2$ and 62ºC to *sul*2, respectively. The amplicons from *sul*2 gene was randomly chosen and submitted to sequencing in an ABI 3130 XL Genetic Analyzer (Applied Biosystems) automated DNA sequencer to determine the primers specifity. The *smf*-1 gene, coding to type-1 fimbriae, was detected by PCR as previously described modifying $MgCl_2$ to 2.5 mM and anneling temperature to 53°C.[8] Amplifications were performed in Veriti Thermal Cycler (Applied Biosystems). The products were analyzed by electrophoresis in agarose gels stained with 0.5 μg/mL ethidium bromide and visualized under UV radiation.

Plasmid profile analysis

The plasmid profile analyses were performed in the isolates resistant to trimethoprim-sulfamethoxazole. The bacterial plasmid DNA was obtained using alkaline lysis protocol[32] with an additional chloroform treatment. The DNA was analyzed by electrophoresis on 0.6% agarose gel stained with 0.5 μg/mL ethidium bromide and visualized under UV radiation. *E. coli* V517 and *E. coli* R55 were used as reference culture for determine the size of the plasmids.

Statistical analysis

The results obtained for the antimicrobial resistance were analyzed using the SPSS software version 15.0 by the descriptive analysis. The data were analyzed and compared by Fisher's exact test and Student´s *t* test when appropriate, for the related samples, considering p value <0.05 statistically significant for all tests.

Results

S. maltophilia was isolated in twenty-eight (3%) of the 936 samples collected from nosocomial environment, of which 19 (67.9%) were from ICU and 9 (32.1%) from the non-ICU unit. The strains of *S. maltophilia* isolated from the ICU were found in 5 (26.3%) bed sides, 4 (21.1%) bidets, 2 (10.5%) supplying balloons, 2 (10.5%) valves of oxygen, air and vacuum, 2 (10.5%) infusion pump, 2 (10.5%) surface of the medical preparation car, 1 (5.3%) stethoscope and 1 (5.3%) monitor of mechanical ventilator. In the rooms from non-ICU unit, the bed sides were also the most frequent site contamined with *S. maltophilia*, 5 isolates (55.6%), followed by 1 (11.1%) valve of oxygen, air and vacuum, 1 (11.1%) infusion pump, 1 (11.1%) stethoscope and 1 (11.1%) table for patient support. *S. maltophilia* was not found in any of the 36 samples collected from the floor. All 28 environmental isolates biochemically compatible with *S. maltophilia* presented a specific amplification product in the PCR targeting the 23S rRNA gene, confirming the *S. maltophilia* identification.

Antimicrobial susceptibility of the *S. maltophilia* strains was determined against three antimicrobial agents by disk-diffusion method. A total of 81 (81%) clinical and 23 (82.1%) nosocomial environmental isolates were susceptible to the three antibiotic tested. The resistance to TMP/SMX was detected in 14 (14%) clinical and 5 (17.9%) environmental *S. maltophilia* isolates. Low percentages of resistance were found for levofloxacin, 9 (9%) and 2 (7.1%) in clinical and environmental strains, respectively. All isolates were susceptible to minocycline. The resistance to all drugs tested showed no difference between the groups of clinical and nosocomial environmental isolates ($p>0.05$).

All *S. maltophilia* strains were shown to be susceptible to chloramphenicol when tested by broth microdilution test. However, 19 (19%) clinical and 6 (21.4%)

environmental strains presented intermediate resistance when analyzed by agar dilution method. Essential agreement rates between broth microdilution and agar dilution were 88% and 92.9% for clinical and environmental isolates, respectively. Although no very major and major errors were observed to choramphenicol, the minor errors rates were 19% for clinical and 21.4% for environmental isolates. The resistance to ceftazidime by agar dilution test was detected in 85 (85%) clinical and 20 (71.4%) environmental isolates, of which 6 (7.1%) and 7 (35%) isolates presented intermediate resistance by the broth microdilution test, respectively. Moreover, 5 isolates, 2 (2%) clinical and 3 (10.7%) environmental showed intermediate resistance when analyzed by agar dilution but were evaluated as susceptible by the broth microdilution test. A total of 16 isolates, 11 (11%) clinical and 5 (17.9%) environmental, was susceptible to ceftazidime, and 2 (2%) clinical strains presented intermediate resistance in both methods (Figure 1). Therefore, essential agreement rates observed between agar dilution and microdilution broth to ceftazidime were 92.9% and 71.4% for clinical and environmental isolates, respectively. Moreover, major and minor errors rates were observed to ceftazidime, 6% and 2% for clinical and 25% and 10.7% for environmental isolates, respectively. Significant difference ($p<0.05$) was observed for the results of minimal inhibitory concentration to ceftazidime and chloramphenicol when both methods were compared.

All 14 clinical and 5 environmental TMP/SMX-resistant *S. maltophilia* strains analyzed carried the *sul*1 gene. None of the isolates presented only the *sul*2 gene and the co-existence of *sul*1 and *sul*2 genes was detected in 6 (42.8%) clinical and 3 (60%) environmental isolates (Table I). The *sul*2 sequence analysis showed 99% of similarity with other *sul*2 sequences deposited in GenBank (EU395473, EU395480 and FJ200242). The presence of the class 1 integrase (*intI*1) gene was detected in 11 (11%) clinical and 3 (10.7%) environmental isolates. Therefore, the presence of integrons was

not detected in 3 (21.4%) clinical and 2 (40%) environmental *sul*1 positive isolates. All isolates that presented specific amplification using a degenerate primer pair for detection of the genes from the integrases *intI*1, *intI*2 and *intI*3 were positives to the presence of class 1 integron. None TMP/SMX-susceptible *S. maltophilia* isolates showed to harbour class 1, 2 and 3 integrons.

Among the 31 isolates carrying *smf*-1, 23 (74.2%) were clinical and 8 (25.8%) environmental, being 9 (39.1%) and 5 (62.5%) isolates with resistance to TMP/SMX, respectively.

Two different plasmid profiles were identified in the 19 *S. maltophilia* isolates resistant to TMP/SMX. Each profile presented only one plasmid, approximatly 7.3 kb or 10 kb size. Fourteen isolates, 9 clinical and 5 environmental presented a 7.3 kb plasmid, of which 6 and 3 isolates, respectively carried the *sul*2 gene. Moreover, 5 clinical strains that presented the *intI*1 gene and carried just the *sul*1 gene contained a plasmid of approximately 10 kb size.

Discussion

S. maltophilia is an emerging multidrug-resistant opportunistic pathogen responsible for several nosocomial infections,[33-37] and presents the ability to form biofilm and survive in environment.[6-8,38] In this context, a total of 936 samples were collected from different sites of nosocomial environment in order to evaluate the levels of presence of this microorganism and characterize the isolates detected. The results obtained indicate the presence of *S. maltophilia* in 3% of the samples analyzed. The contamination rates found in the nosocomial environment were lower than those described by other authors (10.7%, 23% and 17.2%)[39-41] that evaluated samples associated to water and equipments related with cystic fibrosis patients, such as tracheal

tube, ventilator inspiratory circuit, temperature sensor and nebulizers.[39-41] However, our study is the first to describe the identification of *S. maltophilia* in bidet, bed sides, valves of oxygen, air and vacuum, infusion pump, surface of the medical preparation car, stethoscope and monitor of mechanical ventilator, which not necessarily were involved in the care of cystic fibrosis patients. The level of presence of *S. maltophilia* in the hospital environment was also lower than those commonly described to other non-fermentative bacilli, such as *Acinetobacter baumannii* and *Pseudomonas aeruginosa*.[42-45] This result was expected, since these pathogens are usually able to survive a longer period in the environment and are more frequently found in nosocomial infections.

The antimicrobial susceptibility was evaluated for all isolates. Two different methodologies were used to evaluate the minimal inhibitory concentration to ceftazidime and chloramphenicol showing statistically different results. The agar dilution test exhibited higher rates of resistance for both antimicrobial agents tested when compared to broth microdilution method. Discrepant results have also been reported by Girardello et al. (2012)[46] when evaluating the activity of polymyxin B by Etest and disk diffusion compared to the microdilution against *A. baumannii* and *P. aeruginosa*. These authors suggest that the difference between the rates of susceptibility may not be related to the method, but to the culture medium. The evaluation of different brands and lots of Mueller-Hinton agar demonstrated that the cation concentrations vary greately and may not be in accordance with the CLSI guidelines. Therefore, Mueller-Hinton medium should be adjusted for reliable test results since high or low cation concentrations may result in false resistance or false susceptibility, respectively. The high rates of reduced susceptibility to ceftazidime found are according to data previously reported,[47-50] indicating that this antimicrobial agent presents low activity when used as monotherapy to treat *S. maltophilia* infections.

A total of 14.8% of isolates was resistant to TMP/SMX, what corroborates the data reported from other countries[50, 51] and indicates that the levels of resistance to this combination of drugs in Latin America have increased in the last decade, although less dramatically.[52, 53] The main mechanism of sulfamethoxazole resistance in *S. maltophilia* is associated with the *sul* genes expression, but the presence of these genes in this microorganism has not been investigate very frequently and their prevalences are similar when comparing reports.[21-23, 54] In the majority of the 19 TMP/SMX-resistant isolates it was detected the *sul*1 gene and the class 1 integron, 78.6% and 60% of clinical and hospital environmental isolates, respectively. These data corroborate the findings of Barbolla et al. (2004)[21] and Toleman et al. (2007)[22], which demonstrate that the presence of *sul*1 in *S. maltophilia* is associated with class 1 integrons. However, the *sul*1 gene was detected in 5 class 1 integrase negative isolates that showed resistance to TMP/SMX. Hu et al.[23] reported similar results when analyzing clinical isolates and suggested that it may be explained by the possibility that class 1 integrons may not possess identical 5' or 3' ends, what would probably have led to a failure of class 1 integrons detection, since *sul*1 is usually considered as part of this integron. Another alternative explanation that may be speculated is that *sul*1 can have been transferred to another location in the bacterial genome in place of class 1 integrons. The presence of *sul*2 was detected in 9 isolates, contributing to sulfametoxazole resistance, as already described by other authors,[22, 23] but unlikely as reported by Song et al.,[54] which showed that none of the *S. maltophilia* isolates analyzed carried *sul*2 gene. The co-existence of different *sul* genes, although not usually described in the literature, was verified in 9 isolates. Similar data was observed by Hu et al. [23] that showed the co-presence of *sul*1 and *sul*2 genes in 7 clinical strains. Moreover, our data demonstrated a high association of the *sul*2 gene and the presence of a 7.3 kb plasmid. This combination was verified in

all TMP/SMX-resistant isolates that carried *sul*2 gene. Furthermore, 5 *sul*1 positive and class 1 integron negative isolates also showed the 7.3 kb plasmid profile, what reinforces the possibility that the *sul*1 can be carried by a plasmid, as the 7.3 kb plasmid, outside an integron, what could facilitate the increase of the sulfametoxazole resistance by horizontal transfer.

The results described here demonstrate that both clinical and environmental isolates analyzed possessed high rates of susceptibility to minocyclin and levofloxacin. Therefore, the results suggest the possibility of minocycline and levofloxacin represent alternative therapies, as monotherapy or in combination with other antimicrobial agents to the treatment of infection due to TMP/SMX resistant *S. maltophilia*.[20, 33, 55, 56] Similar results have been described by other authors that also found higher susceptibility levels to minocycline and levofloxacin *in vitro* against *S. maltophilia*.[23, 50, 57-59] However, in our knowledge, there are no published data to comprove the efficacy *in vivo* of these antibiotics in *S. maltophilia*.

To evaluate the virulence potencial of *S. maltophilia*, the strains were characterized for the presence of the gene enconding type-1 fimbriae that have been implicated both in biofilm formation and adhesion to abiotic surfaces, such as medical implants and catheters resulting in colonization and infection.[5, 7, 60] The *smf*-1 gene was detected in 31 strains, being 23 clinical and 8 nosocomial environmental isolates. Lower numbers of clinical *S. maltophilia* isolates harboring *smf*-1 gene were detected, when comparing with other studies.[7, 8] The absence of the SMF-1 in the majority of the isolates may be related to the antimicrobial susceptibility presented by the isolates to the majority of tested drugs, since the biofilm formation would confer natural protection against different antimicrobial agents.

In summary, our data demonstrate the presence of *S. maltophilia* in nosocomial environment, suggesting that the hospital environment can act as reservoir of this microorganism, which indicates the need to adopt additional measures for disinfection. In addition, hospital environmental isolates resistant to TMP/SMX were described, which can constitute great concern if it indicates a tendancy to increase and spread. In this sense, it was showed the importance of the *sul* genes in the sulfamethoxazole resistance of *S. maltophilia* and the probable presence of these genes in mobile genetic elements, which may facilitate horizontal transfer, increasing the resistance rates against this antimicrobial agent.

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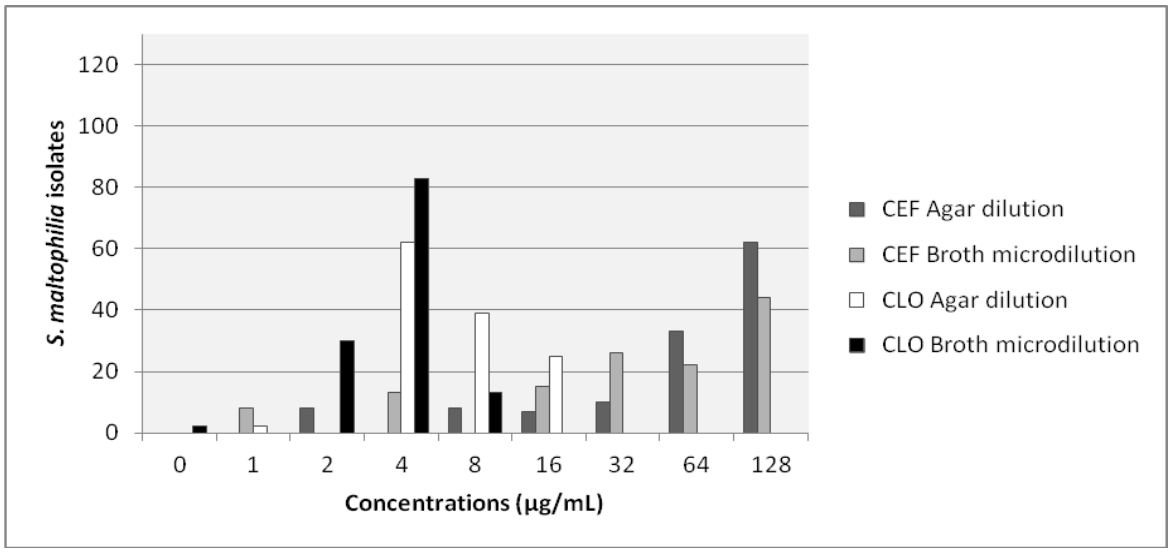

Figure 1. Minimal inhibitory concentration to ceftazidime and chloramphenicol determined by agar dilution and broth microdilution in *Stenotrophomonas maltophilia* isolated from clinical and nosocomial environmental samples. CEF: Ceftazidime; CLO: Chloramphenicol; Susceptible: ≤ 8 µg/ mL; Intermediate: 16 µg/ mL; Resistant: ≥ 32 µg/ mL (CLSI, 2012)

Table I. Characterization of *Stenotrophomonas maltophilia* trimethoprim-sulfamethoxazole resistant isolates in relation to plasmid profile and presence of class 1 integron, *sul*1, *sul*2 and *smf*-1 genes.

Isolate	Origin	Class 1 integron	*sul*1	*sul*2	*smf*-1	Plasmid profile (kb)
3	tracheal aspirate	+	+	-	-	10[a]
23	tracheal aspirate	-	+	-	+	7.3
24	urine	-	+	-	+	7.3
32a	ascites fluid	+	+	-	-	10[a]
39	tracheal aspirate	+	+	+	+	7.3
47	tracheal aspirate	+	+	-	-	10[a]
49	catheter tip	+	+	-	-	10[a]
80	urine	+	+	-	-	10[a]
92	pleural fluid	+	+	+	+	7.3
93	tracheal aspirate	+	+	+	+	7.3
102	blood culture	-	+	-	+	7.3
103	tracheal aspirate	+	+	+	+	7.3
111	tracheal aspirate	+	+	+	+	7.3
112	tracheal aspirate	+	+	+	+	7.3
74	bed sides	+	+	+	+	7.3
146	bed sides	-	+	-	+	7.3
150	stethoscope	+	+	+	+	7.3
761	supplying balloon	+	+	+	+	7.3
775	supplying balloon	-	+	-	+	7.3

[a] Approximate plasmid size.

Capítulo 4

Considerações Finais

4.1 Considerações finais

A identificação correta de *S. maltophilia* é de extrema importância uma vez que este microrganismo é um patógeno oportunista e emergente, podendo permanecer viável no ambiente hospitalar e constituir risco para a infecção continuada de diferentes pacientes. Além disso, este patógeno apresenta resistência intrínseca a uma gama importante de antimicrobianos, dificultando o tratamento de infecções ocasionadas pelo mesmo (66, 67, 68).

Limitações na identificação correta de *S. maltophilia* através de técnicas clássicas de cultivo e de técnicas moleculares têm sido descritas (15, 31, 32, 37, 38, 40, 41, 43). Tais dificuldades são atribuídas à variabilidade genética apresentada por este organismo e à semelhança fenotípica e genotípica existente entre as espécies do gênero *Stenotrophomonas*, que geram confusão de *S. maltophilia* com outras espécies deste gênero, bem como com outros bacilos Gram-negativos não fermentadores (44). Desta forma, foi desenhado um par de oligonucleotídeos iniciadores tendo como alvo o gene 23S rRNA, que foi utilizado para o desenvolvimento de um método de detecção específico para *S. maltophilia* através de PCR. As condições de amplificação para o gene 23S rRNA foram padronizadas para a PCR convencional e mostram-se adequadas também para serem utilizadas na PCR em tempo real, demonstrando que os oligonucleotídeos iniciadores podem ser utilizados para a detecção de *S. maltophilia* através das duas técnicas. A validação do protocolo desenvolvido foi realizada com culturas de referência de *S. maltophilia* e de diferentes bactérias relacionadas, bem como com 100 isolados clínicos previamente identificados através de técnicas clássicas de cultivo e identificação, ocorrendo 100% de concordância entre o sistema bioquímico automatizado Vitek e a PCR.

A identificação de *S. maltophilia* no ambiente hospitalar também foi realizada através do referido protocolo, obtendo-se um total de 3% de amostras positivas para este microrganismo. Este dado difere daqueles relatados em outros estudos que também avaliaram a presença de *S. maltophilia* em diferentes locais do ambiente hospitalar, tais como amostras de água e, principalmente, materiais contaminados com secreções do trato respiratório provenientes de pacientes com fibrose cística (8, 31, 69, 70). No entanto, cabe ressaltar que existe uma considerável associação da colonização por *S. maltophilia* em pacientes com fibrose cística, o que favoreceria um maior isolamento deste microrganismo neste tipo de amostra (71, 72, 73). Desta forma, a comparação dos dados de isolamento obtidos neste estudo com outros trabalhos fica limitada, pois avaliamos locais e equipamentos hospitalares ainda não analisados quanto à presença deste microrganismo, conforme a literatura consultada. Por outro lado, este trabalho é pioneiro no relato da presença de *S. maltophilia* em locais como: ambu, bidê, bomba de infusão, carrinho para a preparação de medicamentos, estetoscópio, válvula de oxigênio, ar e vácuo, mesa de suporte para o paciente e monitor do equipamento de ventilação mecânica, sendo as laterais das camas dos leitos, incluindo as grades de proteção e botões para ajustar altura e posição dos pacientes, o local identificado com maior prevalência (35,7%) de *S. maltophilia*.

Os dados encontrados demonstram que a *S. maltophilia* pode ser capaz de sobreviver no ambiente hospitalar, dessa forma, este ambiente pode ser considerado uma possível fonte de contaminação das mãos e/ou equipamentos dos profissionais da área da saúde. Para tanto, é necessário que seja realizada a correta desinfecção dos equipamentos e materiais utilizados nos hospitais, bem como que seja realizada a correta anti-sepsia das mãos dos profissionais da saúde com o intuito de que os mesmos não sejam carreadores destes patógenos.

Através da caracterização da resistência a drogas antimicrobianas em isolados de *S. maltophilia* foi verificado um alto número de isolados com suscetibilidade reduzida à ceftazidima e suscetíveis ao cloranfenicol. Estes dados corroboram aqueles obtidos por outros estudos que também avaliaram a atividade destas drogas *in vitro* (65, 74, 75, 76). Neste estudo, estes resultados foram obtidos através da determinação da CIM a estas duas drogas pelo método de diluição em agar, bem como por microdiluição, tendo sido observada diferença estatística significativa entre os resultados obtidos pelas duas técnicas. Resultados discrepantes também já foram reportados por Girardello e colaboradores. (2012) (77), ao avaliar a atividade da polimixina B por Etest e difusão de disco comparando com a microdiluição em isolados de *A. baumannii* e *P. aeruginosa*. Os autores sugerem que a diferença encontrada entre as taxas de suscetibilidade não estão relacionadas com o método empregado, mas sim com a concentração de cátions presente nos diferentes meios de cultura analisados.

A identificação de um total de 14,8% de isolados resistentes à associação entre trimetoprim e sulfametoxazol, a qual é considerada a droga de escolha para o tratamento de infecções ocasionadas por *S. maltophilia*, está de acordo com os dados já reportados em outros países (65, 78). Entretanto, pudemos observar um percentual maior de isolados resistentes a estas drogas do que os relatados por outros trabalhos com isolados na América Latina na última década (60, 79), o que pode ser considerado um indicativo de possível aumento de resistência a esta combinação, mesmo que não muito expressivo. Este dado pode constituir preocupação, uma vez que os principais determinantes de resistência a estas drogas podem ser carreados por elementos genéticos móveis, o que enfatiza a necessidade de utilização consciente desta droga para evitar a seleção de cepas resistentes.

A presença dos genes *sul*1 e *sul*2, considerados principais responsáveis pela resistência de *S. maltophilia* ao sulfametoxazol, foi avaliada nos isolados resistentes a TMP/SMX. O gene *sul*1 foi detectado em todos os isolados resistentes a TMP/SMX, corroborando com dados reportados por outros autores (57, 58, 59). Além disso, a maioria dos isolados com este perfil apresentaram concomitantemente o gene *sul*1 e o integron de classe 1, o que era esperado uma vez que este gene é associado com a extremidade 3' desta classe de integron (57, 58). A presença do gene *sul*2 foi verificada em um percentual elevado de isolados, corroborando com dados reportados por Hu et al (2011) (59). Entretanto, é importante salientar que não existem muitos estudos que detectaram a presença deste gene em isolados de *S. maltophilia*, sendo este o primeiro estudo a identificar o gene *sul*2 em isolados do Sul do Brasil. A análise do perfil plasmidial realizada nos isolados resistentes a TMP/SMX indicou que todos os isolados de *S. maltophilia* que carreavam o gene *sul*2, bem como aqueles em que não foi detectado o integron de classe 1, mas apresentaram o gene *sul*1, continham o plasmídio de 7,3 kb, reforçando a associação do gene *sul*2 com este plasmídeo, bem como sugerindo que o gene *sul*1 também possa estar relacionado ao plasmídeo encontrado e, desta forma, ser transmitido através de tranferência horizontal. A co-existência dos genes *sul*1 e *sul*2 é pouco reportada na literatura (59) e esta associação não parece estar relacionada ao aumento da taxa de resistência a TMP/SMX, entretanto, neste estudo, a co-existência entre estes genes foi evidenciada em 47,4% dos isolados resistentes a TMP/SMX analisados.

A verificação de taxas elevadas de suscetibilidade à levofloxacina e à minociclina sugere a possibilidade destes antimicrobianos serem utilizados como terapias alternativas para o tratamento de infecções ocasionadas por *S. maltophilia*,

especialmente naquelas situações em que as infecções forem causadas por isolados resistentes à combinação TMP/SMX (19, 51, 62, 70).

Além disso, foi avaliada a presença do gene *smf*-1 em todos os isolados. Este gene codifica para a fímbria SMF-1, a qual é caracterizada como um fator de virulência associado à produção de biofilme e à adesão a materiais sintéticos. Este estudo detectou um percentual baixo do gene *smf*-1 entre os isolados de *S. maltophilia*, quando comparado com dados já reportados por outros autores (28, 68).

Os dados encontrados neste estudo demonstraram a presença de *S. maltophilia* em materiais e equipamentos hospitalares, o que sugere que o ambiente nosocomial pode atuar como reservatório deste microrganismo, o que evidencia a necessidade de adotar medidas adicionais de desinfecção para evitar a permanência deste patógeno no ambiente. Além disso, foi detectada resistência a TMP/SMX em isolados de *S. maltophilia*, o que pode constituir grande preocupação devido a esta combinação ser a terapia de escolha para infecções causadas por *S. maltophilia*.

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Anexo 1

Guia para autores

Memórias do Instituto Oswaldo Cruz

The manuscript should be prepared using standard word processing software and should be printed (font size 12) double-spaced throughout the text, figure captions, and references, with margins of at least 3 cm. The figures should come in the extension tiff, with a minimum resolution of 300 dpi. Tables and legends to figures must be submitted all together in a single file. Figures, must be uploaded separately as supplementary file.

The manuscript should be arranged in the following order:

Running title: with up to 40 characters (letters and spaces)

Title: with up to 250 characters

Author's names: without titles or graduations

Intitutional affiliations: full address of the corresponding author only

Summary: up to 200 words (100 words in case of short communications). It should emphasize new and important aspects of the study or observations.

Key words: 3-6 items must be provided. Terms from the Medical Subject Headings (Mesh) list of Index Medicus should be used.

Sponsorships: indicating the sources of financial support and change of address

Introduction: should set the purpose of the study, give a brief summary (not a review) of previous relevant works, and state what new advance has been made in the investigation. It should not include data or conclusions from the work being reported.

Materials and Methods: should briefly give clear and sufficient information to permit the study to be repeated by others. Standard techniques need only be referenced.

Ethics: when reporting experiments on human subjects, indicate whether the procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional or regional) and with the Helsinki Declaration of 1975, as revised in 1983. When reporting experiments on animals, indicate whether the institution's or a national research council's guide for, or any national law on the care and use of laboratory animals was followed.

Results: should be a concise account of the new information discovered, with the least personal judgement. Do not repeat in text all the data in the tables and illustrations.

Discussion: should be limited to the significance of the new information and relate the new findings to existing knowledge. Only unavoidable citations should be included.

Acknowledgements: should be short and concise, and restricted to those absolutely necessary.

References: must be accurate. Only citations that appear in the text should be referenced. Unpublished papers, unless accepted for publication, should not be cited. Work accepted for publication should be referred to as "in press" and a letter of acceptance of the journal must be provided. Unpublished data should only be cited in the text as "unpublished observations", and a letter of permission from the author must be provided. The references at the end of the paper should be arranged in alphabetic order according to the surname of the first author.

In the text use the authors surname and date:

Lutz (1910) or (Lutz 1910)

With two authors it is:

(Lutz & Neiva 1912) or Lutz and Neiva (1912)

When there are more than two authors, only the first is mentioned:

Lutz et al. (1910) or (Lutz et al. 1910).

At the end of the paper use the following styles:

Journal article
Chagas C, Villela E 1922. Forma cardiaca da tripanosomiase americana. *Mem Inst Oswaldo Cruz 14*: 15-61.

Book and Thesis
Forattini OP 1973. *Entomologia Médica. Psychodidae, Phlebotominae, Leishmaniose, Bartonelose*, Vol. IV, Edgard Blucher, São Paulo, 658 pp.

Morel CM 1983. *Genes and Antigens of Parasites. A Laboratory Manual*, 2nd ed., Fundação Oswaldo Cruz, Rio de Janeiro, xxii + 580 pp.

Mello-Silva CC 2005. *Controle alternativo e alterações fisiológicas em Biomphalaria glabrata (Say, 1818), hospedeiro intermediário de Schistosoma mansoni Sambom, 1907 pela ação do látex de Euphorbia splendens var. hislopii N.E.B (Euphorbiaceae)*, PhD Thesis, Universidade Federal Rural do Rio de Janeiro, Seropédica, 85 pp.

Chapter in book

Cruz OG 1911. The prophylaxis of malaria in central and southern Brasil. In R Ross, *The Prevention of Malaria*, John Murray, London, p. 390-398.

Journal article on the Internet

Abood S. Quality improvement initiative in nursing homes: the ANA acts in an advisory role. Am J Nurs [serial on the Internet]. 2002 Jun [cited 2002 Aug 12];102(6):[about 3 p.]. Available from: http://www.nursingworld.org/AJN/2002/june/Wawatch.htm

Monograph on the Internet

Foley KM, Gelband H, editors. Improving palliative care for cancer [monograph on the Internet]. Washington: National Academy Press; 2001 [cited 2002 Jul 9]. Available from: http://www.nap.edu/books/0309074029/html/.

Homepage/Web site

Cancer-Pain.org [homepage on the Internet]. New York: Association of Cancer Online Resources, Inc.; c2000-01 [updated 2002 May 16; cited 2002 Jul 9]. Available from: http://www.cancer-pain.org/.

Part of a homepage/Web site

American Medical Association [homepage on the Internet]. Chicago: The Association; c1995-2002 [updated 2001 Aug 23; cited 2002 Aug 12]. AMA Office of Group Practice Liaison; [about 2 screens]. Available from:http://www.ama-assn.org/ama/pub/category/1736.html

DATABASE ON THE INTERNET

Open database:

Who's Certified [database on the Internet]. Evanston (IL): The American Board of Medical Specialists. c2000 - [cited 2001 Mar 8]. Available from: http://www.abms.org/newsearch.asp

Closed database:

Jablonski S. Online Multiple Congenital Anomaly/Mental Retardation (MCA/MR) Syndromes [database on the Internet]. Bethesda (MD): National Library of Medicine (US). c1999 [updated 2001 Nov 20; cited 2002 Aug 12]. Available from:

http://www.nlm.nih.gov/mesh/jablonski/syndrome_title.html

Part of a database on the Internet

MeSH Browser [database on the Internet]. Bethesda (MD): National Library of Medicine (US); 2002 - [cited 2003 Jun 10]. Meta-analysis; unique ID: D015201; [about 3 p.]. Available from: http://www.nlm.nih.gov/mesh/MBrowser.html Files updated weekly. Updated June 15, 2005

Illustrations: figures and tables must be understandable without reference to the text.

Figures: presented in tiff format with a minimum of 300 dpi and photographs must be sharply focused, well contrasted, and if mounted onto a plate, the figures should be numbered consecutively with Arabic numbers. Magnification must be indicated by a line or bar in the figure, and referenced, if necessary in the caption (e.g., bar = 1 mm). Plates and line figures

should either fit one column (8 cm) or the full width (16.5 cm) of the page and should be shorter than the page length to allow inclusion of the legend. Letters and numbers on figures should be of a legible size upon reduction or printing. A colour photograph illustrates the cover of each issue of the Journal and authors are invited to submit illustrations with legends from their manuscript for consideration for the cover

Tables: should supplement, not duplicate, the text and should be numbered with Roman numerals. A short descriptive title should appear above each table, with any explanations or footnotes (identified with *a*, *b*, *c*, etc.) below.

Technical Notes: Technical Notes should communicate rapidly single novel techniques or original technical advances. The entire note should occupy no more than three printed pages including figures and/or tables (it means around 10 double-spaced typed Word file maximum). The text must not be not divided into sections. Therefore, the state of art must be very briefly presented; results must be rapidly presented and discussed at a time. Complementary tables and figures may be published as supplementary data. References must be limited to few essential ones and cited at the end of the note, using the same format as in full papers. A brief summary and three key words must be provided.

Short communications: should communicate rapidly single results or techniques. They should occupy no more than three printed pages including figures and/or tables. They should not contain excessive references. References should be cited at the end of the paper using the same format as in full papers. A brief summary and three key words must be provided.

Alternative format: manuscripts may be submitted following the "Uniform Requirements for Manuscripts Submitted to Biomedical Journals" produced by the International Committee of Medical Journal Editors also known as the Vancouver Style. In this case, authors should follow the guidelines in the fifth edition (Annals of Internal Medicine 1997; 126: 36-47, or at the website http://www.acponline.org/journals/resource/unifreqr/htm) and will be responsible for modifying the manuscript where it differs from the instructions given here, if the manuscript is accepted for publication.

Authors should also follow the Uniform Requirements for any guidelines that are omitted in these Instructions.

Once a paper is accepted for publication, the authors must provide:

An affidavit, provided by the Editorial Office, signed by all authors. Authors from different countries or institutions may sign in different sheets containing the same basic statement; a copyright assignment form, provided by the Editorial Office, signed by the corresponding

author. Page charges: there will be no page charges. Proofs: one set of page proofs will be supplied for the author to check for typesetting accuracy, to be returned by the stipulated date. No changes to the original manuscript will be allowed at this stage.

Anexo 2

Guia para autores

Journal of Hospital Infection

1.1 About the Journal

The *Journal of Hospital Infection* is the scientific publication of the Healthcare Infection Society (formerly known as the Hospital Infection Society). Although HIS is UK based, the JHI is an international publication, and all papers should be of potential relevance to an international audience.

1.2 Scope of the Journal

The JHI focuses on healthcare-associated infection in both community and hospital settings. For example:-

- Outbreak prevention in hospital or community settings
- Healthcare-associated infection surveillance
- Methods of prevention of healthcare-associated infection
- Prevention of infection in immunosuppressed patients
- Infection hazards associated with medical devices
- Role of medical equipment in healthcare-associated infection
- Disinfection and sterilization
- Cleaning, environmental contamination and its surveillance
- Management of clinical waste
- Laboratory diagnostics in relation to infection prevention and control
- Use of antibiotic prophylaxis in infection prevention
- Use of IT systems in infection surveillance
- Design of hospitals and healthcare premises
- Infection hazards associated with critical care units, or other specific healthcare departments

Papers whose main focus is on antimicrobial chemotherapy or antibiotic resistance mechanisms be better suited to a journal like the*Journal of Antimicrobial Chemotherapy*.

Outbreak reports

we welcome these, but there should be something new about them, e.g with a new organism, associated with a new piece of equipment, a new way of dealing with it, or have a definite message.

Audits

should have a clear message or learning point associated with them.

1.3 Article types

The Journal invites articles of the following types:

Full-length, original research articles

Should contain up to a maximum of 4000 words, which includes the structured summary, text, acknowledgements and references. Each figure and/or tables counts as 200 words towards the total. Separate Figures or Tables labelled 1A, 1B, 1C etc would count as three separate tables, not one.

Reviews

We welcome reviews, but please check the topic with the office first, before you spend hours on this. Reviews still go through a peer review process. Reviews should contain up to 5000 words

and up to 150 references. This limit does not apply to national guidelines and may be waived at the Editor's discretion depending on the topic. Suitable review articles will be required to provide a few questions and answers for Continuing Professional Development (CPD).

Letter to the Editor
Letters should contain up to 700 words and no more than 5 references. Letters should not contain structural headings nor a summary. The correspondence section will include letters discussing topics raised by papers already published either in the Journal of Hospital Infection or elsewhere, or could be a *Eureka* type of new idea. Letters will not normally be peer-reviewed, but may be shown to the authors of the article being commented on, who will be invited to respond, should they wish to.

Case reports
Case reports are not normally published unless they illustrate some exceptional point in the field of infection control. When published, case reports usually appear as a short report.

Short reports
should contain up to 1500 words, no more than two small figures or tables, and up to ten references, to take up no more than two printed pages of the journal. The same format should be used as for a full length article, ie Introduction or background/methods/results/discussion and conclusion. The article should begin with an unstructured abstract (ie no subheadings) of up to 100 words only. The same rules apply for declaring conflicts of interest & funding, and also getting a signed agreement from all listed authors. Short reports will be subject to the same peer review process as full-length articles.

2. Format of articles
Please note that it is the authors' responsibility to get the manuscript into the required format before submission. Papers that are submitted with references or other features that do not comply with these instructions will be returned to their authors and will not be considered for publication until they have been resubmitted.

Title Page
This should show the title, names of all authors (but not their degrees) and the name of the institution or department where the work was done, as well as the name, address, telephone and email address of the author to whom the proofs and correspondence should be sent. A running title not exceeding 40 characters and spaces should be provided on the title page.

Summary
This should explain briefly what was done, what was observed and what was concluded. Summaries should be structured, with the following headings:

- Background
- Aim
- Methods
- Findings
- Conclusion

Summaries must not exceed 250 words.
This is arguably the most important part of the entire paper, and will be the first, and perhaps the only, part of your paper that is read.

Keywords
Authors should provide Keywords from their summary; listing them immediately after the summary.

Text
Headings and subheadings may be used in the text. Footnotes should be avoided. All pages of the manuscript should be numbered consecutively in the order: title page, text, references, tables, figures, legends.

Introduction
A brief statement outlining the purpose and context of the paper, but leaving discussion for the Discussion section.

Methods
Preliminary results may be included in the Methods section if necessary.

Results
A statement of results, without discussion of their significance or relationship to those of others. Information may be conveyed in text or in figures or tables but not in both.

Discussion
Do not introduce new results here. Include any weaknesses or limitations of the study.

Acknowledgements
Authors should acknowledge help received in carrying out the work, including supply of bacterial strains, permission to study patients, phage or biotyping of strains, according to accepted custom.

Funding
When the work included in a paper has been supported by a grant or supplies from any source, including a manufacturer or commercial company, this must be indicated. It will be printed at the end of the article.

References
References should be set out in line with the 'Vancouver' style. For a full explanation of this see the Br Med J 1988; 296: 401–405.

Please note that it is the direct responsibility of the authors rather than the Editorial team to list the references accurately, and in the right order and format in the first place.

In the text, references must be consecutively numbered in the order in which they are first mentioned, and must be identified by superscript arabic numerals, after punctuation, e.g. 'as noted by Smith.[4]' References are better placed at the end of sentences so that they don't break up the flow.

The quoted references should be listed in numerical (not alphabetical) order at the end of the article. References cited in tables or in figure legends should be numbered sequentially according to the first mention in the text of the particular table or illustration.

Lists of up to six authors should be fully listed. For seven or more authors list the first three and add *et al.* The journal title (not the article title) should be italic font and the volume number should be shown in bold font.

Journal references should be set out as below:

Elizabeth T. Houang, I.S. Lovett, F.D. Thompson *et al*. *Nocardia asteroides* infection—a transmissible disease. *J Hosp Infect* 1980; **1**: 31–40

C. J. Noble, P. Morgan-Capner, M. Hammer, C. Sivyer, P. Wagstaff, J.R. Pattison. A trial of povidone iodine dry powder spray for the prevention of infusion thrombophlebitis. *J Hosp Infect* 1980; **1**: 47–45

Titles of journals should be abbreviated in accordance with *Index Medicus* (see list printed annually in the January issue of *Index Medicus*).

Article in press
Please include the digital object identifier (DOI) as in the following examples:

1. Russell AD, McDonnel G. Concentration: a major factor in studying biocidal action. *J Hosp Infect* 2000; **44**: 1–3. doi:10.1053/jhin.1999.0654.

2. Jacobsson B-M, Hijelte L, Nystyröm B. Low level of bacterial contamination of mist tents used in home treatment of cystic fibrosis patients. *J Hosp Infect* 2000. doi:10.1053/jhin.1999.0658.

Web addresses
www addresses must **not** be used as references. Please use the DOI for a permanent web article.

Books and chapters
Washington JA, Barry AL. Dilution test procedures. In: Lennette EH, Spaulding EH Truant JP, Eds. *Manual of Clinical Microbiology*, 2nd edn. Washington, DC: American Society for Microbiology 1979; 410–417.

Tables
Tables should be numbered in Roman numerals (e.g. Table III). Each table should be on a separate sheet after the references and should include a title which makes the meaning clear without reference to the text. Use '-' for 'no observation', or 'not measured'.

Figures
Illustrations should be in finished form suitable for reproduction, as large or larger than the final size on the page. Photographs should have strong contrast and be trimmed to exclude unnecessary background. Figures should be planned to fit the proportions of the Journal pages, and details should be easily discriminated at the final size. Colour photographs will be considered only if essential.

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A short explicit legend must be provided for each figure. All such legends should be listed together in the final section of the manuscript.

Bacterial nomenclature
Organisms should be referred to by their scientific names according to the binomial system. When first mentioned the name should be spelt in full and written in italics. Afterwards the genus should be abbreviated to its initial letter, e.g. '*S. aureus*' not '*Staph. aureus*'. If abbreviation is likely to cause confusion or render the intended meaning unclear the names of microbes should be spelt in full. Only those names which were included in the Approved List of Bacterial Names, *Int J Syst Bacteriol* 1980: **30**: 225–420 and those which have been validly

published in the *Int J Syst Bacteriol* since 1 January 1980 have standing in nomenclature. If there is good reason to use a name that does not have standing in nomenclature, the names should be enclosed in quotation marks and an appropriate statement concerning the nomenclatural status of the name should be made in the text (for an example see *Int J Syst Bacteriol* 1980; **30**: 547–556). When the genus alone is used as a noun or adjective, use lower case roman not underlined, e.g.'organisms were staphylococci' and 'acinetobacter infection'. If the genus is specifically referred to, use italics, e.g. 'organisms of the genus *Staphylococcus*'. For genus in plural, use lower case roman e.g. 'salmonellae'; plurals may be anglicized e.g.'salmonellas'. For trivial names, use lower case roman e.g. 'meningococcus'.

Numbers and measurements

Numbers one to nine are written unless they are measurements (e.g. 5 mL). Numbers greater than nine are spelled out if they begin a sentence, or when clarity requires it. Numbers above and including 10 000 have a space, not a comma. A decimal point is preceded by a number or cypher, e.g. '0.5'. Decimal points in columns should be aligned vertically.

Measurements may be expressed in SI or non-metric units. Use 10 mL/h rather than $^{-1}$ or per. When referring to microbial concentrations use expressions such as '10^{x}', not 'x log10'. When referring to changes in microbial concentration, use expressions such as 'reduced by a factor of 10^{x}', not 'reduced by x log10'; 'a log10 reduction factor of x' may also be used.

Statistics

P values and confidence intervals should be included where appropriate. The name and version of any statistical computer package should be written out in full.

Abbreviations

Use capitals for: MIC, MBC, WBC, RBC, DNA, RNA, Group A, B etc. for antigenic or other groups, HPA, CDSC, CDC, WHO, CSF, MSU, EMU, CSU. Use cfu, pfu, mm, m, min, h, in, ft, g, kg, mL, L, im, iv, iu, *P* (probability). Use sp. and spp. (species, singular and plural). Use Gram's stain and Gram-negative bacillus.

Drugs

These should be referred to by their approved generic names. Do not use the proprietary name, as this may vary between countries.

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Dates are usually provided in full, e.g. 11th September 2001. Otherwise, use European Date Format, i.e. 11/9/2001, not 9/11/2001.

Additional points to note

- Use two carriage returns to end headings and paragraphs.
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The Editor retains the customary right to make changes in style and language without consultation.

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The language of the JHI is **British** English.

Please adjust your spell checker if necessary. British spellings include diarrhoea, *Haemophilus*, haematology, paediatrics, leucocyte, leukaemia, bacteraemia, sulphonamides, aetiology. Please note the journal uses UK 'z' spelling (e.g., colonizes).

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Please remember that the JHI is an international journal, and must be readily understood and accessible for readers all over the world. Readers overseas complain sometimes that the JHI comes across as too NHS-focused.

Please avoid using specifically English NHS-jargon, which includes terms like-**NHS Trust** (or even worse, **NHS Foundation Trust**) which would not necessarily conjure up the idea of a hospital to someone based in Berlin or Bogota. "Hospitals Trust" or even just "Hospital/s" should be used instead. If you have to put in the name of your institution, make it clear what you are talking about, e.g.*"The Fitzherbert NHS Foundation Trust is a district hospital with 600 beds, including acute medical, surgical (general and orthopaedic), elderly care, women's services and intensive care".*

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Studies from England compiled as result of Freedom of Information Requests must be clearly indicated as such, and justified. Please note that such studies may often be considered too parochial and specific to England to be suitable for publication in an international journal. You must declare how the data were gathered, and the participating institutions listed (and thanked) in the acknowledgment section. Please note that such papers can cause considerable resentment amongst reviewers and readers alike.

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Contributions should be submitted online at http://jhi.edmgr.com and should conform to the format as set out below. A submission check list can be found here.

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Please note that the JHI has no full-time staff other than the Editorial Coordinator. The Editor and Assistant Editors all have busy hospital or academic jobs. There may be unavoidable delays if we receive an unusually high number of submissions and at peak holiday times, including the second half of December, July and August.

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In common with many other journals, we have two systems of review, internal and external.

External review is where the paper is sent out to one or more external reviewers for comment. We ask for reviews to be returned within 28 days, but this isn't always possible.

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