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Stenotrophomonas maltophilia isolated from patients exposed to invasive devices in a university hospital in Argentina: molecular typing, susceptibility and detection of potential virulence factors

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Abstract

Purpose. The aim of this work was to investigate the presence of selected potential virulence factors, susceptibility and clonal relatedness among 63 *Stenotrophomonas maltophilia* isolates recovered from patients exposed to invasive devices in a university hospital in Argentina between January 2004 and August 2012.

Methodology. Genetic relatedness was assessed by enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) and pulsed-field gel electrophoresis (PFGE). Isolates were characterized by antimicrobial resistance, the presence and/or expression of potential virulence determinants, and virulence in the *Galleria mellonella* model.

Results/Key findings. ERIC-PCR generated 52 fingerprints, and PFGE added another pattern. Resistance to trimethoprim–sulfamethoxazole (6.35 %), levofloxacin (9.52 %) and ciprofloxacin (23.80 %) was detected. All isolates were susceptible to minocycline. All isolates were lipase, protease and siderophore producers, while all but Sm61 formed biofilms. However, 11/63 isolates did not amplify the major extracellular protease-coding gene (*stmPr1*). Sm61 is an *stmPr1*-negative isolate, and showed (as did Sm13 and the reference strain K279a) strong proteolysis and siderophore production, and high resistance to hydrogen peroxide. The three isolates were virulent in the *G. mellonella* model, while Sm10, a low-resistance hydrogen peroxide *stmPr1*-negative isolate, and weak proteolysis and siderophore producer, was not virulent.

Conclusion. This is the first epidemiological study of the clonal relatedness of *S. maltophilia* clinical isolates in Argentina. Great genomic diversity was observed, and only two small clusters of related *S. maltophilia* types were found. Minocycline and trimethoprim–sulfamethoxazole were the most active agents. *S. maltophilia* virulence in the *G. mellonella* model is multifactorial, and further studies are needed to elucidate the role of each potential virulence factor.

INTRODUCTION

Stenotrophomonas maltophilia, a widespread environmental non-fermentative Gram-negative bacillus, is an emerging global opportunistic pathogen. Even though *S. maltophilia* is not a highly virulent pathogen, it has recently been classified as one of the leading drug-resistant pathogens in hospitals worldwide by the World Health Organization [1]. Infection occurs principally in immunocompromised subjects and in patients exposed to invasive devices and/or broad-spectrum antibiotics. *S. maltophilia* can cause pneumonia associated with mechanical ventilation and the use of nebulizers, catheter-related bacteraemia, septicaemia, haemodialysis and intravenous line-related infections, and urinary tract infection [2]. The incidence of *S. maltophilia* hospital-acquired infections is increasing, and is associated with crude mortality rates ranging from 14 to 69 % in patients with bacteraemia. Respiratory tract colonization is seen in cystic fibrosis (CF) patients with increasing frequency; nevertheless, there is controversy as to whether this leads to a poorer clinical outcome [2].

Despite the broad spectrum of clinical syndromes associated with *S. maltophilia* infections, little is known about its

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Keywords: *Stenotrophomonas maltophilia*; invasive devices; molecular typing; susceptibility; biofilms; potential virulence factors.

Abbreviations: CAS, chrome azurol S; CF, cystic fibrosis; CLSI, Clinical and Laboratory Standards Institute; ERIC-PCR, enterobacterial repetitive intergenic consensus PCR; ICU, intensive care unit; Nar, membrane-bound nitrate reductase; PFGE, pulsed-field gel electrophoresis; StmPr1, major extracellular protease; SXT, trimethoprim–sulfamethoxazole.

virulence factors [3]. The factors that could be involved in the virulence of *S. maltophilia* include, among others, extracellular enzymes such as DNase, lipases, proteases (especially the major extracellular protease StmPr1) and siderophores [4–7]. An important virulence factor of *S. maltophilia* is its capacity to adhere to biotic or abiotic surfaces, such as those of medical devices, and to form biofilms [8–10]. Although *S. maltophilia* is an obligate aerobe, the membrane-bound nitrate reductase (codified by *nar* genes) that supports growth in the absence of oxygen with nitrate as a terminal electron acceptor is present in some strains. The potential for growth under micro-oxic conditions may enhance the pathogenicity of this organism by increasing its ability to grow in biofilms [11].

S. maltophilia biofilms exhibit phenotypic characteristics that are distinct from those of planktonic organisms, including increased resistance to antimicrobial compounds [12–14]. Treatment of *S. maltophilia* infections is difficult because of its intrinsic multi-resistance to antibiotics, including carbapenems, and its capacity to form biofilms [2, 15]. Trimethoprim–sulfamethoxazole (SXT) has long been regarded as the first-line therapeutic drug for the therapy of *S. maltophilia* infections. However, the global emergence of SXT resistance has been reported and is related to the spread of dihydropteroate synthase (*sul1* and *sul2*) genes, which are usually located on mobile genetic elements [16–18]. Other drugs with activity against *S. maltophilia* are minocycline and newer fluoroquinolones, such as moxifloxacin and levofloxacin [19].

Epidemiological studies revealed a great diversity among *S. maltophilia* isolates. Gherardi *et al.* [20] recently reviewed the most common genotyping methods used for clinical epidemiology of *S. maltophilia* isolates.

The aim of this work was to investigate the presence of selected potential virulence factors, susceptibility and clonal relatedness among 63 *S. maltophilia* isolates recovered from patients exposed to invasive devices in a university hospital in Argentina between January 2004 and August 2012.

METHODS

Bacterial isolates and culture conditions

A total of 63 *S. maltophilia* isolates recovered from 60 non-cystic fibrosis patients exposed to invasive devices between January 2004 and August 2012, were prospectively collected from the microbiology laboratory at the Hospital de Clínicas José de San Martín, Universidad de Buenos Aires, Argentina. Among the 63 isolates, only 6 from the respiratory tract corresponded to 2 sequential isolates from each of 3 patients (Sm56 and Sm57, Sm59a and Sm59b, and Sm60a and Sm60b). The isolation sites are indicated in Table 1. The isolates were identified as *S. maltophilia* by using conventional methodology and API 20NE (BioMérieux) according to the manufacturer's instructions. The identification was confirmed by matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS), performed in duplicate using a Bruker Daltonics MicroFlex LT instrument and Biotyper 3.1 software (Bruker Daltonics) as previously described [21]. *S. maltophilia* K279a, whose genome has been fully sequenced (GenBank: AM743169.1), was used as a reference strain [11]. Isolates were kept frozen at −20 °C in 15 % glycerol. Before use, bacteria were cultured on tryptone soya agar (TSA; Oxoid Ltd) for 24 h at 35 °C. Unless otherwise stated, all cultures were grown in tryptone soya broth (TSB; Oxoid Ltd) and incubated for 24 h at 35 °C. When required, the cultures were vigorously aerated on a gyratory water bath shaker (model G75, New Brunswick Scientific Co.) at 200 r.p.m.

Susceptibility

The antimicrobial susceptibility for SXT, ciprofloxacin and levofloxacin was determined retrospectively using Phoenix AST panels (NMIC-406BD Diagnostics) following the manufacturer's recommendations. The antimicrobial susceptibility for minocycline was determined by disk diffusion according to Clinical and Laboratory Standards Institute (CLSI) recommendations [22]. The minimum inhibitory concentrations (MICs) of levofloxacin and ciprofloxacin were also assessed by the broth microdilution method using cation-adjusted Mueller–Hinton broth (Laboratorios Britania). *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as quality control strains. The breakpoints used were those recommended by the CLSI for *S. maltophilia* [22]. Since there are no specific CLSI criteria for ciprofloxacin, the criteria for *P. aeruginosa* were used.

Pulsed-field gel electrophoresis (PFGE)

Agarose gel plugs containing chromosomal DNA were digested overnight at 37 °C with 15 U of the restriction enzyme *XbaI* (Thermo Fisher Scientific). Electrophoresis was performed using a CHEF-DRII system (BioRad) over 20 h at 14 °C, with 5 to 35 s of linear ramping at 6 V cm^{-1}. Gels were stained with ethidium bromide and digitized (Molecular Imager Gel Doc XR, Bio-Rad). Band patterns were compared using the criteria of Tenover *et al.* [23]. The dendrogram of PFGE profiles was constructed using UPGMA cluster analysis with Treecon software (http://bioinformatics.psb.ugent.be). The strains were defined as having a clonal relationship if they possessed ≥85 % similarity to the PFGE profiles [24].

Enterobacterial repetitive intergenic consensus (ERIC) PCR

Genomic DNA from each of the 63 isolates was obtained by the boiling method. Amplification reactions were performed in a 25 µl final volume containing 2 U of *GoTaq* DNA polymerase (Promega) in 5× *GoTaq* reaction buffer, 4 mM $MgCl_2$, dimethyl sulfoxide 10 %, 0.4 mM of each dNTP, 50 pmol of primer ERIC-2 (5′-AAGTAAGTGACTGGGGTGAGCG-3′, Thermo Fisher Scientific) and 3 µl of bacterial DNA. The samples were amplified in a thermocycler (Biometra TPersonal). The amplification profile was 2 min at 94 °C, 30 cycles of 30 s at 94 °C, 1 min at 50 °C and 4 min at 72 °C, followed by a final extension of 10 min at 72 °C.

Table 1. Sources, API 20NE biocodes and potential virulence factors of *S. maltophilia* isolates

S. maltophilia isolates	Isolate source	API 20NE Biocode*	Nitrate reduction	Presence of *narG gene*	Proteolysis	Presence of *stmPr1* gene	Oxidative stress resistance	Siderophore production
Sm K279a	Blood	I	+	+	+++	+	+++	+++
Sm 9	Urine§	I	+	+	++	+	++	++
Sm 10	Tracheal aspirate	II	−	−	+	−	+	+
Sm 11	Urine§	II	−	+	+	+	+	+
Sm 13	Blood‖	I	+	+	+++	+	+++	+++
Sm 14	Renal biopsy¶	I	+	+	+++	+	++	++
Sm 15	Peritoneal fluid#	I	+	+	+	+	++	+
Sm 17	Blood‖	I	+	+	++	−	++	++
Sm 18†	Blood¶	I	+	+	++	−	++	+
Sm 19†	BAL	III	+	+	+++	+	++	+
Sm 20†	BAL	II	−	−	++	−	+++	++
Sm 26†	BAL	I	+	+	+++	+	++	++
Sm 27†,‡	BAL	I	+	+	+	+	+++	+++
Sm 28	BAL	II	−	−	+	+	+	++
Sm 29†	Tracheal aspirate	I	+	+	+++	+	+	++
Sm 30	Blood‖	II	−	−	+++	+	+++	+++
Sm 31†	Tracheal aspirate	I	+	+	+++	+	+	++
Sm 32	Tracheal aspirate	I	+	+	+++	+	+	++
Sm 33	Blood‖	I	+	+	+++	+	++	++
Sm 34	Blood‖	II	−	−	+++	+	++	+
Sm 35	Tracheal aspirate	II	−	−	++	+	++	+
Sm 36†,‡	BAL	II	−	−	++	+	++	++
Sm 37†	Tracheal aspirate	II	−	−	+	+	+++	++
Sm 38†	BAL	II	−	−	+++	+	+++	++
Sm 39†	BAL	I	+	+	+++	+	+++	++
Sm 40	BAL	III	+	+	++	+	+++	+++
Sm 41†,‡	Tracheal aspirate	I	+	+	++	+	++	+++
Sm 42‡	Tracheal aspirate	II	−	−	++	+	++	++
Sm 43	Tracheal aspirate	II	−	−	++	−	++	++
Sm 44†	Blood‖	I	+	+	+++	+	++	+
Sm 45†,‡	BAL	I	+	+	++	+	++	++
Sm 46†	Tracheal aspirate	II	−	−	+++	+	++	+
Sm 47†	BAL	I	+	+	+	−	+++	+
Sm 48†	Tracheal aspirate	I	+	+	++	+	+++	+
Sm 49†	Tracheal aspirate	I	+	+	++	−	+++	++
Sm 50†	Tracheal aspirate	I	+	+	+++	+	+	++
Sm 51†	Tracheal aspirate	II	−	−	+++	+	++	++
Sm 52†	Tracheal aspirate	II	−	−	++	+	++	++
Sm 53†	Tracheal aspirate	I	+	+	+++	+	++	++
Sm 54†	Tracheal aspirate	III	−	+	+++	+	+	++
Sm 55†	Tracheal aspirate	I	+	+	++	+	++	+
Sm 56‡	Tracheal aspirate	I	+	+	+++	+	+++	++
Sm 57†	Tracheal aspirate	I	+	+	+++	+	++	++
Sm 58†	Blood‖	I	+	+	+++	+	+	++
Sm 59a†	Tracheal aspirate	II	−	−	+++	+	++	++
Sm 59b†	Tracheal aspirate	II	−	−	++	+	++	++
Sm 60a†	Tracheal aspirate	II	−	−	++	+	++	++
Sm 60b†	BAL	II	−	−	++	+	++	++
Sm 61†	Blood‖	II	−	−	+++	−	+++	+++
Sm 62†	Tracheal aspirate	I	+	+	+++	+	++	+++
Sm 63†	Blood‖	II	−	−	++	+	++	+++

Table 1. cont.

S. maltophilia isolates	Isolate source	API 20NE Biocode*	Nitrate reduction	Presence of *narG gene*	Proteolysis	Presence of *stmPr1* gene	Oxidative stress resistance	Siderophore production
Sm 64†	Tracheal aspirate	I	+	+	++	+	++	+++
Sm 65†	Tracheal aspirate	II	−	−	++	+	++	++
Sm 66†	Tracheal aspirate	I	+	+	+++	+	+	++
Sm 67†	Tracheal aspirate	I	+	+	+++	+	++	+
Sm 68†	Blood‖	II	−	−	+++	+	++	++
Sm 69	Tracheal aspirate	I	+	+	+++	+	+++	++
Sm 70	Tracheal aspirate	I	+	+	+++	+	+++	+
Sm 71	Tracheal aspirate	II	−	−	+++	−	++	+
Sm 72†	Tracheal aspirate	II	−	−	+++	−	++	+
Sm 73†	Tracheal aspirate	III	+	+	+++	+	++	+
Sm 74†	Blood‖	I	+	+	++	−	+	++
Sm 75	Tracheal aspirate	II	−	−	++	+	++	++
Sm 76†	Blood‖	II	−	−	++	+	+++	+

*API20NE biocodes were determined based on the ability to reduce nitrate to nitrite and to assimilate citrate: I, 1472341 (+/+); II, 0472341 (−/+); and III, 1472340 (+/−).

†*S. maltophilia* isolates from polymicrobial cultures.

‡Colonization. Sm K279a, isolated from the blood of an oncological patient, was used as a reference strain. *S. maltophilia* isolates from patients exposed to invasive devices: mechanical ventilation [broncho alveolar lavage (BAL) and tracheal aspirates].

§Urinary catheter.

‖Vascular catheter.

¶Haemodialysis catheter.

#Peritoneal dialysis catheter.

Nitrate reduction, presence of *narG* and *stmPr1* genes, proteolysis, oxidative stress resistance and siderophore production were determined and expressed as described in the Methods section.

A negative control was run with each experiment. The amplification products were analyzed by electrophoresis in a 1.5 % agarose gel with a 1-Kb DNA ladder (Thermo Fisher Scientific) as a size marker and were detected after ethidium bromide staining. The PCR product patterns were digitized and compared visually. They were considered to be identical when the positions of all the bands matched, regardless of band intensity [25].

Detection of *narG* and *stmPr1* genes

Genomic DNA was obtained as described above. The primers for the amplification of the *narG* gene (forward: 5′-GGCTTGAGCACGATGCGGGT-3′, reverse: 5′-GTGGGCAAGGAGCACGAGGC-3′) and *stmPr1* gene (forward: 5′-CGTGCCAGCTTCTCCAACTA-3′, reverse: 5′-AGGACTGTTGATGGTGCAGG-3′) were designed in this study on the basis of the genomic sequence of K279a (GenBank: AM743169.1). Amplification reactions were performed in a final volume of 25 µl containing 1 U of *GoTaq* DNA polymerase (Promega) in 5× *GoTaq* reaction buffer, 2.5 mM $MgCl_2$, dimethyl sulfoxide 10 %, 0.4 mM of each dNTP, 10 pmol of the corresponding primers (Thermo Fisher Scientific) and 3 µl of bacterial DNA. The amplification profile was 5 min at 95 °C, 30 cycles of 1 min at 95 °C, 30 s at 50 °C or 48 °C (for *narG* or *stmPr1*, respectively) and 1 min at 72 °C, followed by a final extension of 6 min at 72 °C. The amplification products, 570-bp (*stmPr1*) and 623-bp (*narG*) fragments, were analysed by electrophoresis in a 1.5 % agarose gel.

Exoenzyme production

The proteolytic activity was visualized on nutrient agar (Difco) supplemented with 1 % skim milk powder, and the lipolytic activity was visualized on tributyrin agar base containing 1 % glycerol tributyrate (Merck) as previously described [9]. Clear haloes after incubation for 24 h at 35 °C indicated exoenzyme activity. The proteolytic activity of each isolate was classified as: weak (+, 14–16 mm), moderate (++, >16–18 mm), or strong (+++, >18 mm).

Nitrate reduction

The ability of the isolates to reduce nitrate was assessed by using the API 20NE system as described by Smibert and Kriegand [26].

Siderophore production

The production of siderophores was screened by a modified chrome azurol S (CAS) agar assay [7]. Siderophore production was recorded as the diameter of the orange halo produced by the colony, and each isolate was classified as: weak siderophore producer (+, 4.0–5.9 mm), moderate siderophore producer (++, 6.0–8.9 mm), or strong siderophore producer (+++, >9 mm).

Sensitivity to oxidative stress

Sensitivity to hydrogen peroxide was determined by the disk assay [9]. Sensitivity to H_2O_2 was determined by the zones of growth inhibition surrounding each disk after incubation at 35 °C for 24 h. The resistance of each isolate to hydrogen peroxide was classified as: low (+, >18 mm), moderate (++, 16–18 mm), or high (+++, <16 mm).

Motility assays

Swimming, swarming and twitching agar plates were used to test different types of cell motility as described by Rashid and Kornberg [27].

Biofilm formation assay

Biofilms were prepared using a static microtitre plate model as previously described [13]. After 24 h of incubation at 35 °C, the wells were washed with PBS to remove non-adherent cells and biofilms were stained with 0.01 % crystal violet (CV; Mallinckrodt) for 30 min. The plates were washed, and the dye bound to the biofilm was extracted with ethanol 95 %. The total biomass (attached cells and extracellular matrix) was quantified by measuring the OD_{540} of the dissolved CV using a FlexStation 3 plate reader (Molecular Devices). Each isolate was assayed in octuplicate. Uninoculated medium controls (ODc) were included. The cutoff was defined as three standard deviations above the mean ODc. Each isolate was classified as follows: weak biofilm producer $OD \leq 2 \times ODc$, moderate biofilm producer $2 \times ODc < OD \leq 4 \times ODc$, or strong biofilm producer $OD > 4 \times ODc$ [28].

Galleria mellonella killing assay

The virulence of selected *S. maltophilia* isolates was evaluated by infecting larvae of the wax moth *G. mellonella* as previously described [29]. Caterpillars were incubated in Petri dishes lined with filter paper at 30 °C for 96 h and scored for survival daily. Insects were considered dead when they displayed increased melanization and failed to respond to touch. In all experiments, 12 caterpillars were used for each isolate, including a control group of caterpillars inoculated with physiological saline to monitor for killing due to physical trauma. Experiments that had more than one dead caterpillar in the control group were discarded and repeated. Survival curves were plotted using the Kaplan– Meier method, and differences in survival were calculated using the log-rank test (GraphPad Prism version 5.0, GraphPad Software, www.graphpad.com).

Statistical analysis

All experiments were performed at least in duplicate and repeated on three different occasions. Statistical analysis was performed using GraphPad Prism version 5.0. The results were analysed by one-way analysis of variance (ANOVA) with Dunnett's post test, and differences were considered significant at P values<0.05.

RESULTS

Table 1 shows that most of the 63 local isolates were recovered from respiratory specimens (n=46, 73.02 %) and blood (n=13, 20.63 %), while the remaining 4 were recovered from renal biopsy (1), peritoneal fluids (1) and urine (2). *S. maltophilia* was the only micro-organism recovered from 21 (33.33 %) samples, while polymicrobial cultures were obtained from 42 (66.67 %) samples. The most frequently co-isolated bacteria were *Acinetobacter* spp. (45.24 %) and *Pseudomonas aeruginosa* (28.57 %). *S. maltophilia* was also co-isolated with *Klebsiella* spp. (3/42), *Serratia* spp. (2/42), *Staphylococcus epidermidis* (3/42) and yeasts (3/42) (data not shown). Six patients with positive respiratory cultures were considered to have been colonized by *S. maltophilia* (Table 1), according to the Centers for Disease Control and Prevention definitions [30].

Identification and typing of *S. maltophilia* isolates

Local isolates were identified as *S. maltophilia* using conventional methodology and API 20NE, and the identification was later confirmed by MALDI-TOF MS. As expected, biotyping was poorly discriminative. The whole population of isolates was represented by three API biotypes, based on the ability to reduce nitrate to nitrite and to assimilate citrate (Table 1). In contrast, ERIC-PCR revealed great genetic diversity. Fifty-six different PCR fingerprints were obtained with primer ERIC2, 52 isolates presented unique fingerprints and 11 isolates showed 4 DNA banding patterns shared by 2 (Sm41-Sm45 and Sm56-Sm57), 3 (Sm43-Sm51-Sm52) or four isolates (Sm59a-Sm59b-Sm60a-Sm60b) (Fig. 1). *XbaI*-PFGE was conducted to evaluate the relationship among the indistinguishable isolates, with the exception of two isolates from a neonate, Sm56 and Sm57, which had lost viability. PFGE generated identical patterns for isolates Sm41 and Sm45, as well as for isolates Sm43, Sm51 and Sm52 (Fig. 2). On the other hand, PFGE provided evidence that the profile shared by Sm59a and Sm59b was different from that of isolates Sm60a and Sm60b (Fig. 2).

Susceptibility

Table 2 summarizes the susceptibility data for SXT and fluoroquinolones.The resistance rates were 6.35 % for SXT, 9.52 % for levofloxacin and 23.80 % for ciprofloxacin, while all 63 isolates were susceptible to minocycline. The interpretation of the MICs of levofloxacin and ciprofloxacin assessed by the broth microdilution method was coincident with that from the Phoenix AST panels. It is worth noting that intermediate susceptibility rate for ciprofloxacin was higher than that for levofloxacin (17.46 and 4.76 %, respectively).

Potential virulence factors

S. maltophilia isolates were analysed for the presence and/or expression of the following potential virulence determinants. The results from the 63 *S. maltophilia* isolates and from the reference strain K279a are shown in Table 1.

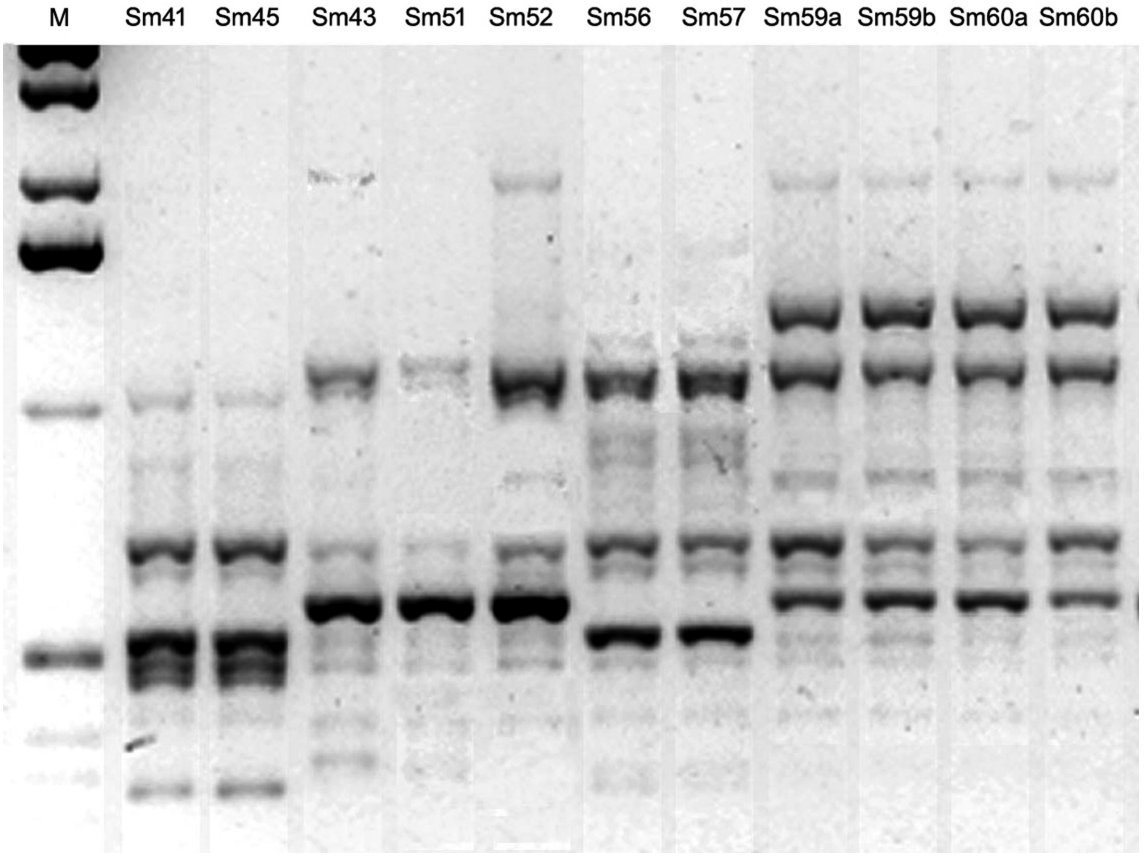

Fig. 1. Representative ERIC-PCR amplification patterns of *S. maltophilia* isolates. M, 1 Kb molecular weight marker.

Motility and biofilm formation

All of the isolates showed the ability to move via swimming and twitching, but we could not detect swarming motility under the experimental conditions used in this study. Microplate assays showed that all but one (Sm61) of the local *S. maltophilia* isolates formed biofilms. Even though they formed strong biofilms, as did K279a, the OD_{546} of the 24 h biofilms stained with CV ranged from 0.900 to 2.300, showing differences in biomass formation ability among isolates (data not shown).

Siderophore production

When grown on modified CAS agar plates, all of the studied isolates were CAS-positive for siderophore production. Eighteen *S. maltophilia* isolates were weak siderophore producers, 36 were moderate siderophore producers and only 9 isolates, as well as K279a, were strong siderophore producers.

Sensitivity to oxidative stress

S. maltophilia isolates that were tested for their sensitivity to hydrogen peroxide using a disk inhibition assay showed diameters of zones of growth inhibition ranging from 14 to 21 mm. Three different groups were defined, which showed high resistance (K279a and 16/63 isolates), moderate resistance (36/63), or low resistance to hydrogen peroxide (11/63).

Protease and lipase activities and detection of the *stmPr1* gene

All isolates were lipase and protease producers. The cultures showed zones of proteolysis ranging from 14 to 22 mm. The proteolytic activity was classified as strong for 32/63 isolates and K279a, moderate for 24 isolates and weak for 7 isolates. As reported in Table 1, even though all of the isolates were protease producers, only 52/63 local isolates, as well as the reference strain K279a, amplified the 570-bp fragment corresponding to the *stmPr1* gene that encodes the major alkaline serine protease by PCR.

Nitrate reduction activity and detection of the *narG* gene

The results obtained by using the technique described by Smibert and Krieg [26] were in agreement with the results for the nitrate reduction test in the API 20NE system. Even though nitrate reduction activity was detected in 35 isolates, the PCR for *narG* was positive for 37/63 isolates, and for K279a. The isolates that were negative in the biochemical tests but positive for the *narG* detection were Sm11 and Sm54.

Virulence of *S. maltophilia* in the *G. mellonella* infection model

The virulence of selected *S. maltophilia* isolates was evaluated by infecting larvae of the greater wax moth

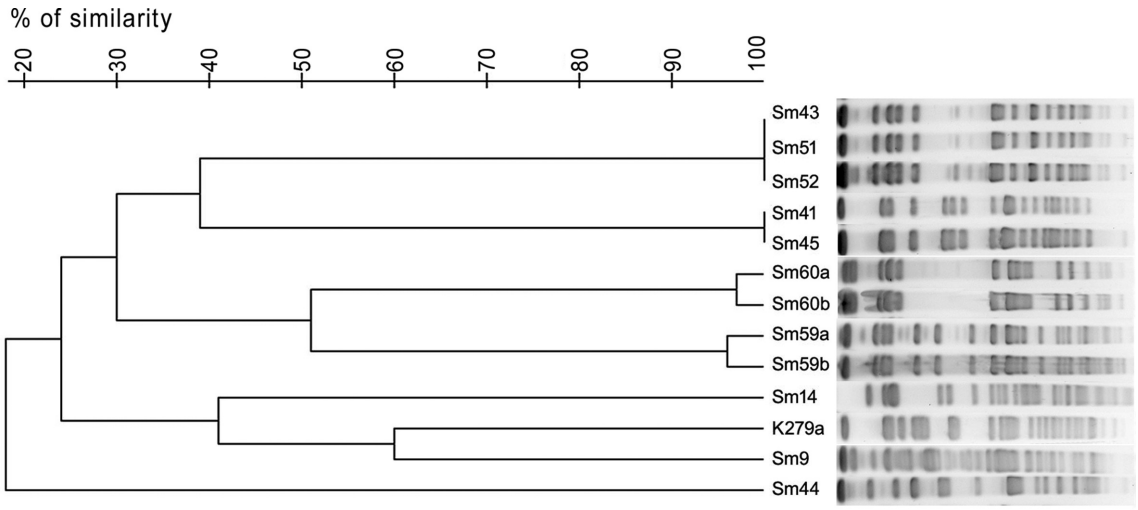

Fig. 2. Phylogenetic analysis of PFGE profiles of *S. maltophilia* isolates. The dendrogram of the PFGE profiles of 12 local *S. maltophilia* isolates and K279a was constructed using UPGMA cluster analysis with Treecon software. Strains were defined as having a clonal relationship if they possessed ≥85 % similarity to the PFGE profiles.

G. mellonella. The killing assays were performed with K279a, Sm10, Sm13 and Sm61, under the conditions that we reported previously [29]. Fig. 3 shows the corresponding survival curves of a single representative trial. The infection of caterpillars with K279a resulted in a death rate of 19 % after 48 h of inoculation, and one of 43 % after 96 h, while Sm13 was able to kill 30 % of the caterpillars after 96 h of inoculation. A different response, but one that was not statistically significant, was obtained with Sm61, which was able to kill 55 % of caterpillars after 48 h of inoculation and reached a death rate of 64 % after 96 h. On the other hand, no dead caterpillars were detected in the group infected with Sm10 or in the control group.

DISCUSSION

Indwelling medical devices are used a great deal in modern medicine, and unfortunately provide access to bacteria. During the period from January 2004 to August 2012, *S. maltophilia* was isolated from 60 patients with device-associated infections at a university hospital in Argentina. In accordance with previous reports, most of the 63 isolates were recovered from respiratory specimens (73.02 %) and blood (20.63 %) (Table 1) [2]. Polymicrobial cultures were obtained from 66.67 % of the samples and, in agreement with previous reports, the most frequently co-isolated bacteria were *Acinetobacter* spp and *P. aeruginosa* [31]. Differentiation between clinical infection and colonization is more difficult when *S. maltophilia* is recovered from mixed cultures [32]. In the present study, a neonate initially considered as colonized because of the low count of *S. maltophilia* in his first tracheal aspirate sample (Sm56, 10^2 c.f.u.) resulted, 9 days later, compatible with nosocomial infection on the basis of the high *S. maltophilia* count (>10^6 c.f.u.) detected in his second sample (Sm57), and died a few days later. Nosocomial colonization and subsequent infection by multiresistant strains of *S. maltophilia* can be considered to be a risk in vulnerable patients, and they have resulted in serious outbreaks [33]. Furthermore, the presence of this micro-organism in polymicrobial cultures

Table 2. Susceptibility of 63 *S. maltophilia* isolates to three antimicrobial agents

Antimicrobial agent	MIC range (μgml^{-1})	Percentage of isolates		
		Resistant	Intermediate	Susceptible
SXT	0.5/9.5–≥2/38*	6.35	–	93.65
Ciprofloxacin	0.25–128†	23.80	17.46	58.74
Levofloxacin	0.25–16†	9.52	4.76	85.72

SXT, trimethoprim–sulfamethoxazole.

*MICs of SXT determined using Phoenix AST panels.

†MICs of fluoroquinolones assessed by the broth microdilution method. Interpretative criteria were applied according to the Clinical and Laboratory Standards Institute guidelines for *S. maltophilia* (SXT and levofloxacin) and *P. aeruginosa* (ciprofloxacin) [22].

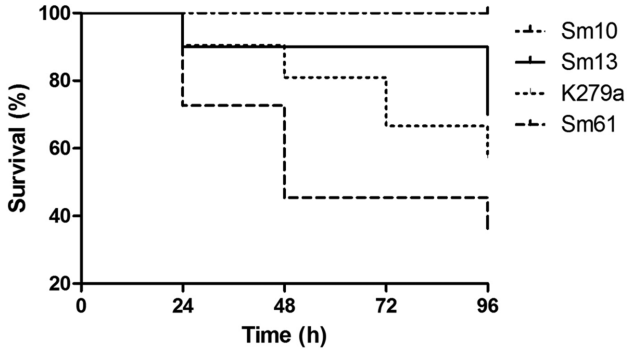

Fig. 3. Virulence of selected *S. maltophilia* isolates in the *Galleria mellonella* infection model. Kaplan–Meier survival curves for *G. mellonella* larva for 96 h after injection with 1×10^5 UFC/larva of Sm10, Sm13, Sm61 and K279a are shown. Each data set corresponds to a single representative trial with the specified isolate (n=12). No dead caterpillars were detected in the control group (not shown).

should be considered, since β-lactamase-packed outer membrane vesicles are capable of establishing extracellular β-lactam degradation [34].

The treatment of *S. maltophilia* is quite difficult, given its intrinsic resistance to a number of antibiotics, and because it is able to acquire new resistance via horizontal gene transfer and mutations [15]. In the present study, the resistance rates were 6.35 % for SXT, 9.52 % for levofloxacin and 23.80 % for ciprofloxacin, while all 63 isolates were susceptible to minocycline. In agreement with previous reports, levofloxacin was more active than ciprofloxacin against *S. maltophilia* isolates (85.72 and 58.74 %, respectively) [13, 35].

It has been reported that phenotypic profiles are not able to discriminate between *S. maltophilia* isolates [32]. Accordingly, biotyping was poorly discriminative (Table 1). In agreement with previous genotyping studies, ERIC-PCR revealed great diversity among the 63 local *S. maltophilia* isolates [32]. Primer ERIC2 generated 52 unique fingerprints, and PFGE under *Xba*I restriction was used for typing indistinguishable isolates (Fig. 1). Isolates Sm41 and Sm45, from two colonized patients hospitalized in February and March 2008, respectively, in the same bed of an intensive care unit (ICU), shared the same PFGE DNA banding pattern (Fig. 2). Recently, Gallo *et al.* [36] reported that *S. maltophilia* was found in the hospital environment, especially on bed rails. PFGE also generated identical patterns for isolates Sm43 (March 2008), Sm51 (July 2009) and Sm52 (August 2009), which were recovered from three patients on different wards (Fig. 2). *S. maltophilia* transmission has been reported, even across wards, and it may survive long periods in hospital settings due to its capacity to form biofilms and colonize humid surfaces [32, 33, 37, 38]. On the other hand, PFGE presented evidence indicating that the profile shared by Sm59a and Sm59b was different from that of the Sm60a and Sm60b isolates (Fig. 2). These four isolates were from two patients hospitalized in different wards in the same period (October 2009). The great genomic diversity observed in this study is in accordance with previous genotyping studies in which most patients harboured unique types and only occasional small clusters of related *S. maltophilia* types were found [39, 40].

To obtain an insight into the virulence of *S. maltophilia*, the local isolates were characterized by the presence and/or expression of selected potential virulence factors. All of the isolates showed the ability to move via swimming and twitching, motilities that have been involved in biofilm formation, which is a well-known virulence factor of *S. maltophilia* [8–10, 41]. Microplate assays showed that all but one (Sm61) of the local *S. maltophilia* isolates formed strong biofilms, although the biomass formation ability of the isolates differed. Accordingly, Flores-Treviño *et al.* [40] examined the biofilm production of non-CF clinical isolates of *S. maltophilia* from two hospitals in Mexico, and found that all of the strains were able to produce biofilms. However, the majority of the isolates were classified as weak and moderate biofilm producers, while only 13.4 % (16/119) of them were categorized as strong biofilm producers. The fact that in our study almost all the isolates (62/63) formed strong biofilms could be associated with their geographical origin and/or with having been collected from device-associated infections.

Siderophores are considered to be important virulence factors for many pathogens because they allow microorganisms to survive in the iron-restricted environment of the host. As shown in Table 1, local isolates were classified as weak, moderate or strong siderophore producers. We had previously reported that 31 local *S. maltophilia* isolates and K279a produced catechol-type siderophores in low-iron media [7]. Furthermore, we found that K279a presented two iron-repressed outer membrane proteins with homology with FepA (enterobactin receptor) and another putative TonB-dependent siderophore receptor [29]. Recently, Nas and Canciotto [4] reported that inactivation of *entC* (enterobactin biosynthesis homologgene) and *fepA* prevented the production and utilization of siderophores, respectively. These authors demonstrated that *S. maltophilia* secretes a novel catecholate siderophore that is distinct from enterobactin.

Susceptibility to oxidative stress, a critical determinant for bacterial survival, was evaluated by the disk assay. More than half of the local *S. maltophilia* isolates showed moderate resistance (57.14 %) to hydrogen peroxide, while 25.40 and 17.46 % exhibited high and low resistance, respectively (Table 1).

All local isolates were lipase and protease producers (Table 1). Our results are in agreement with those reported in a study performed in Brazil [42]. In contrast, in another study that was performed in Japan, lipase production was only observed in 21/66 isolates (31.8 %) [39]. Windhorst

et al. [5] characterized StmPr1, a major extracellular alkaline serine protease that degrades several human proteins from serum and connective tissues and inactivates components of the host immune response. It has been recently reported that K279a encodes a type II secretion system (Xps) that secretes serine proteases StmPr1, StmPr2 and StmPr3, which are responsible for secreted proteolytic activities, as well as for the morphological and cytotoxic effects on a human lung epithelial cell line [43, 44]. The authors also demonstrated that StmPr1 contributes the most to Xps-mediated activities. Even though all local isolates were protease producers, 11/63 isolates did not amplify the 570-bp fragment corresponding to *stmPr1* by PCR. The possible explanations for this result are related to the fact that *S. maltophilia* produces other proteases, or that the primers used in the PCR test were designed on the basis of the genomic sequence of K279a (GenBank: AM743169.1).

As was mentioned above, some strains of *S. maltophilia* have a membrane-bound nitrate reductase [11]. Table 1 shows that nitrate reduction activity was detected in 35 local isolates, while the PCR for *narG* rendered positive results for 37/63 isolates. Sm11 and Sm54 scored negative for nitrate reductase activity, but amplified the 623-bp (*narG*) fragment correctly, suggesting the presence of mutations in this gene or in other genes of the nitrate reductase operon. The potential for growth under microoxic conditions may enhance the pathogenicity of *S. maltophilia* by increasing its ability to grow in biofilms [11]. However, no correlation was observed between nitrate reduction activity and biofilm formation capacity. This lack of correlation could be due to the fact that biofilm formation is a multifactorial process.

Very little is known about the pathogenic mechanisms of *S. maltophilia*. The *G. mellonella* infection model is a useful tool for research on *S. maltophilia* virulence, and one study suggested that StmPr1, rather than StmPr2, could be a relevant virulence factor of *S. maltophilia* [6]. However, the authors mentioned that protease activity is not solely responsible for virulence, as two environmental protease-positive strains exhibited poor killing activity. We have previously determined the role of iron as a signal, likely through the ferric uptake regulator (Fur) system, for *S. maltophilia* biofilm formation and virulence [29]. A spontaneous *fur* mutant was more virulent than its isogenic parental strain K279a in the *G. mellonella* killing assay, possibly due to the pleiotropic effects of this mutation, including increased biofilm formation and MnSOD production. In the present report, the virulence of selected *S. maltophilia* isolates was evaluated by infecting *G. mellonella* larvae. The killing assays were performed with Sm10, Sm13 and Sm61, with K279a as a reference strain (Fig. 3). We had previously shown that Sm10 (tracheal aspirate) and Sm13 (blood) exhibited different biofilm formation capacities on hydrophilic and hydrophobic surfaces [9, 45]. Sm61 (blood) was selected, since it is the only local isolate that does not form biofilms. The infection of caterpillars with K279a and Sm13, which shared similar virulence-related traits, including the presence of the *stmPr1* gene (Table 1), resulted in death rates of 43 and 30 % after 96 h of inoculation, respectively. Surprisingly, Sm61 was able to kill 64 % of caterpillars after 96 h of inoculation. Even though Sm61 is a non-biofilm-forming *stmPr1*-negative isolate, it showed strong proteolysis and siderophore production, and high resistance to hydrogen peroxide, as well as K279a and Sm13. On the other hand, no dead caterpillars were detected in the group infected with Sm10, a *stmPr1*-negative isolate that showed weak proteolysis and siderophore production, and low resistance to hydrogen peroxide. These results indicate that the virulence of the *S. maltophilia* in this model is multifactorial.

This study has some limitations. First, it was only conducted in a single large tertiary university hospital. Second, because of the character of this work, hospital environmental isolates could not be included in the *S. maltophilia* epidemiological analysis. Third, defined mutants of *S. maltophilia* lacking virulence factors were not included in the *G. mellonella* killing assay. *S. maltophilia* virulence in the *G. mellonella* model is multifactorial, and further studies using defined mutations in the virulence factor-encoding genes are needed to fully delineate the individual contributions of these genes to the virulence of *S. maltophilia*.

However, the strength of our investigation is that it is the first epidemiological study of the clonal relatedness of *S. maltophilia* clinical isolates in Argentina. A great deal of genomic diversity was observed, and only two small clusters of related *S. maltophilia* types were found. Our results reinforced the concept that although PFGE is the gold standard for DNA fingerprinting, ERIC-PCR is a useful technique for epidemiological typing in the context of hospital infection control. Furthermore, this study provides new data on potential virulence factors of *S. maltophilia*, collected from patients exposed to invasive devices in a university hospital in Argentina. Finally, our data support the use of minocycline and SXT as first-line therapeutic choices in Argentina.

The increasing frequency of the isolation of *S. maltophilia* worldwide accentuates the need for the continuous monitoring of clonal relatedness, antibiotic susceptibility and the expression of potential virulence factors in clinical and hospital environmental isolates.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement

Patient data were anonymized and only routine diagnostic samples were used in this study. Thus, ethical approval was not required.

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