Contents lists available at SciVerse ScienceDirect

Journal of Global Antimicrobial Resistance

Identification of the first bla_{CMY-2} gene in *Salmonella enterica* serovar Typhimurium isolates obtained from cases of paediatric diarrhoea illness detected in South America

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ARTICLE INFO

ABSTRACT

The objectives of this study were to investigate clinical isolates of *Salmonella enterica* serovar Typhimurium resistant to β-lactam antibiotics, to characterise their mechanisms of antibiotic resistance and to evaluate the possible biological cost of expressing resistance genes. Two oxyimino-cephalosporin-resistant *Salmonella* isolates obtained from children with diarrhoea were characterised. The occurrence of plasmid-encoded bla_{CMY-2} genes was confirmed by molecular methods and conjugation assays; transcription levels were determined by quantitative real-time PCR (qRT-PCR). The genomic context of the β-lactamases, replicon type and addiction systems were analysed by PCR. Genomic relatedness of both isolates was studied by pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) assays. Growth curves, motility and invasiveness assays in Caco-2 cells were performed to analyse the bacterial fitness of both isolates. Both isolates carried a bla_{CMY-2}-like allele in an IncI plasmid and belonged to the same MLST sequence type (ST19); nevertheless, they showed extensive differences in their PFGE profiles and virulotypes. Isolate STM709 appeared to lack the *Salmonella* virulence plasmid and displayed less motility and invasiveness in cultured cells than isolate STM910. qRT-PCR showed that isolate STM709 had higher bla_{CMY-2} mRNA levels compared with STM910. Altogether, the results suggest that a plasmid carrying bla_{CMY-2} could be disseminating among different clones of *S.* Typhimurium. Different levels of bla_{CMY-2} mRNA could have an effect on the fitness of this micro-organism, resulting in lower invasiveness and motility.

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1. Introduction

Salmonella enterica serovar Typhimurium and *S. enterica* serovar Enteritidis are the main serotypes responsible for human salmonellosis worldwide, causing digestive tract infection and invasive infections associated with consumption of contaminated food, especially poultry [1]. In Uruguay, *S.* Typhimurium has been one of the main causes of human salmonellosis since 1971, alternating with *S.* Enteritidis since 1995 [2,3].

Antibiotic treatment for salmonellosis is indicated for life-threatening infections in children, elderly people or immunocompromised patients, and therapeutic recommendations are usually trimethoprim/sulfamethoxazole (SXT), fluoroquinolones and oxyimino-cephalosporins [4].

Therapeutic options for paediatric patients are particularly reduced since resistance to SXT is variable among bacterial enteropathogens and it may even aggravate infections with Shiga toxin-producing *Escherichia coli* [5,6]. Furthermore, paediatric fluoroquinolone usage is restricted on account of possible adverse effects [7].

On the other hand, use of oxyimino-cephalosporins has been reduced in North American countries (Mexico, USA and Canada)

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owing to the increasing occurrence of *Salmonella* spp. strains bearing *bla*$_{CMY-2}$ [8–10].

Until recently, the situation in South America was different on account of the absence of reports on this continent regarding *Salmonella* isolates carrying such β-lactamases.

In Uruguay, *S.* Typhimurium has historically remained susceptible to oxyimino-cephalosporins [11]. The first report of a human isolate of oxyimino-cephalosporin-resistant *S.* Enteritidis (CTX-M-14) was in 2012 [12], with a precedent of a TEM-144-producing *S. enterica* serovar Derby strain isolated from eggs in 2002 [13].

In contrast to extended-spectrum β-lactamase production, the presence of class C β-lactamases in *S.* Typhimurium has been associated with a fitness cost in terms of reduced invasive capability and lower growth rates [14].

The aims of this work were to characterise the mechanisms of resistance to oxyimino-cephalosporins, amoxicillin/clavulanic acid (AMC) and cefoxitin present in two *S.* Typhimurium isolates obtained from children with diarrhoea in Uruguay. In addition, the putative effects of expressing such resistance genes on the fitness of the isolates were evaluated by analysing their growth rate, motility and invasiveness into epithelial cells in comparison with a contemporary isolate susceptible to third-generation cephalosporins.

2. Materials and methods

2.1. Strains

Two faecal isolates of *S.* Typhimurium (STM709 and STM910) were obtained from children with diarrhoea in two tertiary care centres in Montevideo, Uruguay, in July 2009 and September 2010, respectively. *Salmonella* Typhimurium strains SL1344 and STM23 were used as controls. SL1344 is a mouse-virulent, antibiotic-susceptible laboratory strain [14], whereas STM23 is an oxyimino-cephalosporin-susceptible clinical isolate of *S.* Typhimurium collected in Uruguay in 2009 from a case of diarrhoea.

Strains were routinely grown aerobically at 37 °C in Luria–Bertani (LB) broth (Amresco, Solon, OH) supplemented with 100 mg/L ampicillin (Sigma–Aldrich, St Louis, MO) when indicated. Serotyping was conducted at the Centro Nacional de *Salmonella* (CNS) housed in the Departamento de Bacteriología y Virología, Instituto de Higiene, Universidad de la República, Montevideo, Uruguay [11].

2.2. Antibiotic susceptibility testing and plasmidic ampC (p/ampC) detection

Antibiotic susceptibility profiles were determined using a Vitek2® Compact System (bioMérieux, Marcy-l'Étoile, France) and results were interpreted according to Clinical and Laboratory Standards Institute (CLSI) guidelines [15]. Minimum inhibitory concentrations (MICs) for donor and recipient strains and transconjugants were determined by Etest (bioMérieux) according to the manufacturer's instructions.

Bacterial DNA was extracted using a QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany). p/*ampC* alleles were detected and sequenced by multiplex PCR according to Pérez-Pérez and Hanson [16].

2.3. Conjugation assays and plasmid characterisation

Conjugation assays were carried out using a rifampicin-resistant *E. coli* J53-2 strain as recipient. Transconjugants were selected on MacConkey agar plates (Oxoid Ltd., Basingstoke, UK) supplemented with rifampicin (150 mg/L) (Sigma–Aldrich) and ceftriaxone (1 mg/L) (Sigma–Aldrich) [17].

Incompatibility (Inc) groups of *ampC*-carrying plasmids were determined by PCR-based replicon typing according to Carattoli et al. using total DNA from donor strains and transconjugants as template [18].

Conjugative plasmid size was estimated by treatment with S1 nuclease (Fermentas, Life Sciences, Vilnius, Lithuania) followed by pulsed-field gel electrophoresis (PFGE) as described previously [12].

Addiction systems were sought in *ampC*-bearing plasmids by PCR, as reported elsewhere [12].

The link between *bla*$_{CMY-2}$ and different insertion sequences such as IS*Ecp1*, IS*26*, IS*903* and IS*CR1* was sought by PCR using primers previously described by Eckert et al. [19].

2.4. Characterisation of genomic relatedness

2.4.1. Multilocus sequence typing (MLST)

MLST characterisation of strains STM709 and STM910 was conducted following the guidelines described in the *S. enterica* MLST database (http://mlst.ucc.ie/mlst/dbs/Senterica).

2.4.2. Pulsed-field gel electrophoresis

DNA macrorestriction analysis with *Xba*I was performed by PFGE following PulseNet protocols. *Salmonella enterica* serovar Braenderup H9812 was used as a marker [20]. Bearing in mind that only two isolates were analysed, band patterns were interpreted according to the criteria of Tenover et al. [21].

2.5. Virulotyping

PCR detection of genes coding for proteins secreted by type III secretion systems (*avrA*, *sopE*), *Salmonella* virulence plasmid genes (*spvC*, *pefA*), *S.* Typhimurium genomic island CS54 (*shdA*, *sivH*) and phage-encoded genes (*gogB*, *Sb41*) was performed. PCR was performed as previously described using specific primers for *invA* as an internal control [2,22–25].

2.6. Motility tests

Motility tests were performed as described by Yim et al. [26]. Briefly, 2 μL of overnight cultures grown in LB broth were spotted onto the surface of an LB plate containing 0.3% agar and were incubated for 6 h at 37 °C. The diameter of the motility halo was measured after 6 h of incubation. Assays were performed in triplicate and motility was previously confirmed by phase-contrast microscopy of mid-log bacterial cultures grown in LB broth.

2.7. Cell invasion assays

The human colon carcinoma (Caco-2) cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA). Caco-2 cells were maintained in minimal essential medium with Earle's salts (Gentaur, Kampenhout, Belgium) supplemented with 2 mM L-glutamine (Sigma–Aldrich) and 15% foetal bovine serum (PAA, Pasching, Austria) at 37 °C in 5% CO_2, at up to 80% confluence. Caco-2 invasion assays were performed as described previously [26]. Briefly, log-phase bacterial cultures (supplemented with ampicillin for STM709 and STM910 when indicated) were added to the cells at a multiplicity of infection of ca. 30:1, the plates were centrifuged for 5 min at $200 \times g$ and invasion was allowed to proceed for 1 h at 37 °C. The medium was changed to gentamicin-containing medium (100 mg/L) (Sigma–Aldrich) and was incubated for 1.5 h. Then, 20 μL of each supernatant was plated on LB agar to verify that no viable bacteria were remaining and the cells were then washed and lysed with 0.1% Triton X-100 (Sigma–Aldrich) for bacterial release and counting. Data are expressed as the

percentage of the initial inoculum. Each isolate was tested in triplicate in two independent experiments.

2.8. Growth curves

Growth curves for p/ampC-bearing strains were performed. Briefly, overnight cultures in LB broth supplemented with 100 mg/L ampicillin were diluted 1/100-fold in pre-heated LB broth with ampicillin; dilutions were incubated at 37 °C with shaking (200 rpm). Measurements of the optical density at 600 nm (OD_{600}) were taken at regular intervals (30 min) until stationary growth phase. All assays were performed in triplicate.

2.9. Quantitative real-time PCR (qRT-PCR)

Total RNA from bacterial cultures grown to late exponential phase (OD_{600} of ca. 0.9) in LB broth supplemented with ampicillin was extracted using an RNeasy® Protect Bacteria Kit (QIAGEN). Then, 1 μg of the resulting RNA was treated with DNase (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions and was reverse transcribed using M-MLV reverse transcriptase (Invitrogen) and random primers in a 20 μL reaction. Then, 2 μL of a 1/128 dilution of this reaction was used for qRT-PCR using SYBR Green (QuantiTect®; QIAGEN) in a Corbett Rotor-Gene 6000 (QIAGEN). Primer sequences were as follows: icdA-F, 5′-TGGTATCGGTGTTGATGTCACTC-3′; icdA-R, 5′-CATCCTGGCCG-TAAACCTGTGTG-3′; CMY2-F, 5′-CCGCCTGCTGCACTTAGCCA-3′; and CMY2-R, 5′-CCCGGAGTCCATTGCGGCTG-3′. The cycling programme was 15 min at 95 °C and 40 cycles of 15 s at 94 °C, 30 s at 57 °C and 30 s at 72 °C. The comparative threshold cycle (C_T) method was used for relative mRNA quantitation [27]. Briefly, the C_T obtained for bla_{CMY2} was normalised by the C_T obtained for the housekeeping gene icdA (isocitrate dehydrogenase) in each sample, giving the ΔC_T ($\Delta C_T = C_{TicdA} - C_{TCMY-2}$) [28]. Then, $2^{\Delta C_T}$ was calculated in each sample and plotted. Since bla_{CMY-2} is expressed constitutively, $2^{\Delta C_T}$ was plotted instead of the more frequently used $2^{\Delta \Delta C_T}$ or fold change. cDNA from isolate STM23 grown in the same conditions but without ampicillin was used as a negative control.

2.10. Statistical analysis

Differences in motility, growth curves and invasiveness in Caco-2 cells were compared by one-way analysis of variance (ANOVA) using the Bonferroni adjustment as post hoc test (SPSS 17.0; SPSS Inc., Chicago, IL). Homoscedasticity was assessed using Levene's test of homogeneity of variances. The Welch test was used whenever data showed heteroscedasticity. Differences in bla_{CMY-2} mRNA levels were analysed using the Mann–Whitney U-test (GraphPad Prism 4.0; GraphPad Software Inc., La Jolla, CA). A P-value of <0.05 (two-tailed) was considered as statistically significant in all tests.

3. Results

3.1. Antibiotic susceptibility and plasmidic ampC detection

Both isolates (STM709 and STM910) showed resistance to cefotaxime, ceftazidime, cefoxitin and AMC but were susceptible to cefepime, carbapenems, gentamicin, amikacin, nalidixic acid, ciprofloxacin and SXT (Table 1). On the other hand, isolate STM23 was susceptible to all antibiotics tested.

STM709 and STM910 yielded a 462 bp PCR product with primers CITMF/CITMR [16]. Further sequencing confirmed the presence of a bla_{CMY-2}-like gene. In both cases, bla_{CMY-2} was located downstream of ISEcp1 as previously reported [29].

3.2. Characterisation of bla_{CMY-2}-encoding plasmids

Transconjugants were obtained for both isolates, designated TcSTM709 and TcSTM910, respectively. Both transconjugants showed the same resistance patterns as the donor strains (Table 1). The presence of bla_{CMY-2} was confirmed by PCR. Donor strains and transconjugants shared a single ca. 85 kb IncI1 plasmid (pSTM709 and pSTM910) as revealed by PFGE analysis after S1 nuclease digestion (data not shown). The search for addiction systems showed only the presence of pndAC in both conjugative plasmids.

3.3. Characterisation of genomic relatedness

Both strains (STM709 and STM910) belonged to sequence type 19 (ST19) (allelic profile 10, 7, 12, 9, 5, 9, and 2), whereas PFGE analysis showed at least eight different bands between both strains (Fig. 1).

PCR detection of virulence-related genes also yielded significant differences; although both strains harboured avrA, gogB and shdA, STM709 showed positive PCR results for sivH whereas STM910 harboured spvC, pefA, sopE and Sb41 (Table 1).

The absence of spvC and pefA in STM709 suggests that this isolate could be missing the S. Typhimurium virulence plasmid [30].

Table 1
Susceptibility profile and virulotype of strains under study.

	Salmonella enterica serovar Typhimurium strains		Transconjugants		Escherichia coli J53-2	Target
	STM709	STM910	TcSTM709	TcSTM910		
Minimum inhibitory concentration (mg/L)						
CTX	32	16	4	8	0.125	
CAZ	48	32	16	16	0.38	
Replicon type	IncI1	IncI1	IncI1	IncI1	–	
Addiction system	pndAC	pndAC	pndAC	pndAC	–	
Virulotyping						
spvC	–	+	N/D	N/D	N/D	Virulence plasmid
pefA	–	+	N/D	N/D	N/D	
avrA	+	+	N/D	N/D	N/D	SPI-1
sopE	–	+	N/D	N/D	N/D	SopEΦ (SPI-1)
sivH	+	–	N/D	N/D	N/D	CS54 island
shdA	+	+	N/D	N/D	N/D	
gogB	+	+	N/D	N/D	N/D	Gifsy-1
Sb41	–	+	N/D	N/D	N/D	φSE20

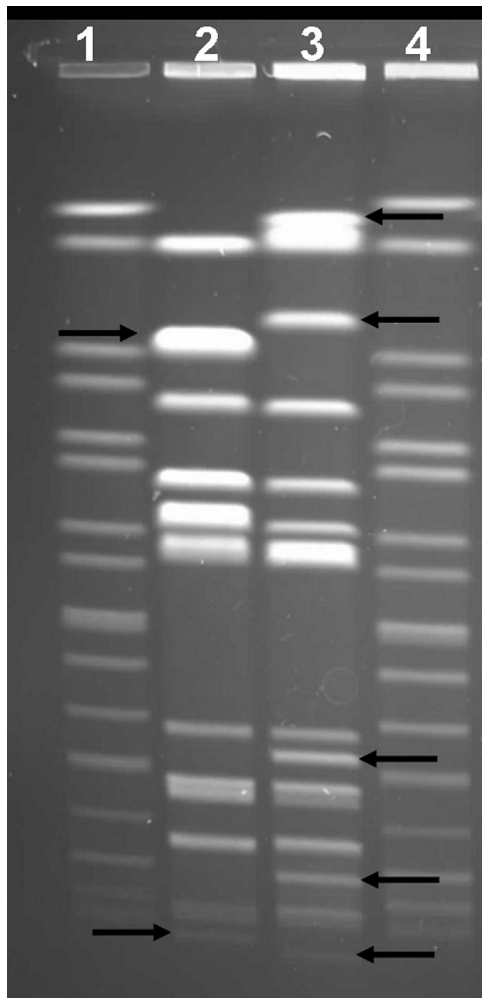CTX, cefotaxime; CAZ, ceftazidime; N/D, not determined.

Fig. 1. Pulsed-field gel electrophoresis (PFGE) analysis of *Salmonella enterica* serovar Typhimurium strains STM709 and STM910. Lanes 1 and 4, reference strain *S. enterica* serovar Braenderup H9812; lane 2, STM709 (bla_{CMY-2}); lane 3, STM910 (bla_{CMY-2}). Arrows indicate differences in band patterns. Variations in band intensity were not considered.

Fig. 2. (A) Results of motility assays on 0.3% Luria–Bertani (LB) agar for *Salmonella enterica* serovar Typhimurium strains STM709 and STM910 and control strains SL1344 and STM23. The mean ± standard deviation of three independent experiments is plotted. (B) Invasiveness in Caco-2 cells. Results are expressed as percentage of the initial inoculum. The mean ± standard deviation of two independent experiments is plotted. (C) Quantitation of bla_{CMY-2} mRNA levels. Total RNA from late log-phase cultures of strains STM709 or STM910 was obtained and the levels of bla_{CMY-2} transcripts were measured by quantitative real-time PCR. The mean ± standard deviation of $2^{\Delta C_T}$ of five replicates in two independent experiments is plotted. *Indicates a statistically significant difference.

3.4. Motility assays and growth curves

Strain STM709 was significantly less motile than strain STM910 and reference strains SL1344 and STM23, indicated by a halo of motility ca. 80% smaller after 6 h of incubation in semisolid agar (Fig. 2A). Phase-contrast microscopy of bacterial cultures revealed an aggregated phenotype for strain STM709. Since bacterial clumping could hamper motility, strain STM709 was centrifuged and carefully re-suspended by pipetting to ensure disaggregation of possible clumps prior to plating onto semisolid agar plates. Disaggregation was confirmed by wet-mount microscopy. Motility results were the same with or without previous disaggregation.

Regarding growth curves, strains STM709 and STM910 did not show statistically significant differences (data not shown).

3.5. Cell invasion assays

Caco-2 invasion assays showed that strain STM709 was ca. eight and six times less invasive than reference strains SL1344 and STM23, respectively (see Fig. 2B). Strain STM910 appeared to be more invasive than both reference strains. To corroborate that clumping formation was not interfering with the invasiveness of STM7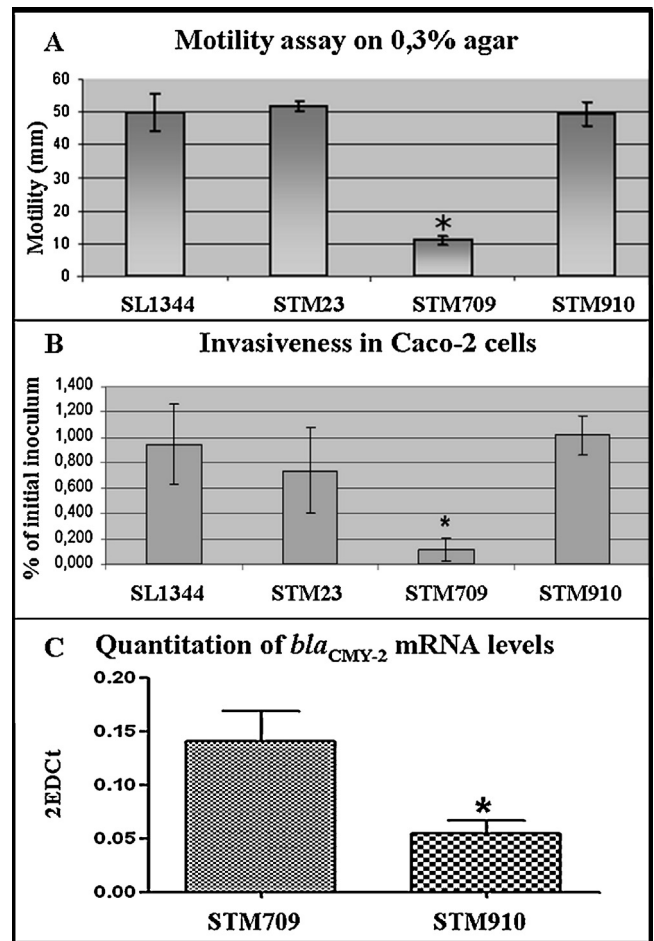09, the bacterial inoculum was centrifuged and re-suspended prior to infecting the monolayer, and bacterial disaggregation was assessed by wet-mount microscopy.

3.6. Quantitative real-time PCR measurement of bla_{CMY-2} mRNA levels

Since isolate STM709 showed reduced motility and cell invasiveness while displaying slightly higher MICs to β-lactam antibiotics compared with isolate STM910, and based on background data reporting reduced fitness in *S.* Typhimurium owing to the expression of a class C β-lactamase [14], we decided to evaluate the levels of bla_{CMY-2} gene expression in both isolates in order to evaluate the putative biological cost of expressing this gene. In line with the previous report, qRT-PCR analysis revealed that the levels of bla_{CMY-2} mRNA were 2.6 times higher in isolate STM709 compared with isolate STM910 (Fig. 2C), this difference being statistically significant.

4. Discussion

Antibiotic susceptibility patterns of *S. enterica* are changing in Uruguay and probably in South America as well. In this sense, we

previously reported the first clinical isolate of *S.* Enteritidis carrying $bla_{CTX-M-14}$ in this continent [12].

Considering that *S.* Typhimurium lacks *ampC* in its chromosome [14] as well as the lack of reports of p/*ampC*-bearing *Salmonella* isolates in South America, such isolates could be misidentified in those clinical laboratories in South America lacking highly discriminative identification capabilities on account of an 'atypical' susceptibility pattern.

From the analysis of two different clinical isolates, results suggest that a plasmid carrying bla_{CMY-2} could be disseminating among different clones of *S.* Typhimurium. Although both isolates belonged to ST19, important differences were found regarding their PFGE patterns and virulence-associated genes, suggesting that both isolates have dissimilar genetic backgrounds. Such differences could be due to the presence/absence of the virulence plasmid, the genetic structure of genomic island CS54 and the presence of phages SopEΦ and φST64b.

Strain STM709 (isolated in 2009) appears to lack the *Salmonella* virulence plasmid as well as SopEΦ and ST64b prophages; besides, it also displays less motility and is less invasive than *S.* Typhimurium strains SL1344 and STM23. Formation of bacterial clumps by STM709 could impair its motility as well as altering the interaction with host cell receptors, thus affecting its invasive capability. Nevertheless no differences were found in motility or invasiveness between the bacterial inocula with or without disaggregation prior to the assays. The features exhibited by STM709 are in accordance with findings by Morosini et al. regarding the biological cost for *S.* Typhimurium of expressing a class C β-lactamase [14].

On the other hand, strain STM910 displayed a similar behaviour to the control strains (i.e. SL1344 and STM23). In this sense, strain STM910 (isolated in 2010) harboured the virulence plasmid as well as the SopEΦ and ST64b prophages, displayed a motile phenotype as well as good invasive capability in Caco-2 monolayers.

Further studies are required to determine whether differences in CMY-2 expression levels are due to a larger pSTM709 copy number or to the presence in strain STM709 of an additional chromosomally encoded bla_{CMY-2} gene [31]. Likewise, more studies are needed to determine (i) whether dissimilar CMY-2 expression levels and/or plasmid-related factors are accountable for the loss of fitness of *Salmonella* Typhimurium and (ii) whether pSTM709 or pSTM910 can be equally carried by clones with different degrees of virulence.

We provide here an initial insight into the nature of a bla_{CMY-2}-encoding plasmid identified in South America and the different characteristics of its hosts.

In conclusion, two isolates of *S.* Typhimurium bearing bla_{CMY-2} in an IncI1 'epidemic resistance plasmid' were studied to the best of our knowledge, this is the first description of *Salmonella* strains carrying such a gene in South America. In addition, the fact that one of the studied isolates displayed a good fitness could become an emerging problem in this continent, reinforcing the relevance of maintaining active vigilance.

Funding

Competing interests

GG is a member of Carrera del Investigador Científico (CONICET, Argentina).

Ethical approval

Not required.

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