Molecular typing, antibiotic resistance profiles and biocide susceptibility in *Salmonella enterica* serotypes isolated from raw chicken meat marketed in Venezuela

Fanny González[1], *María Araque*[2,*]

Abstract

Introduction ***Salmonella*** **is a common bacterial cause of foodborne diarrhea worldwide. The purpose of this study was to characterize antimicrobial resistance and susceptibility to biocides in *Salmonella enterica* serotypes isolated from raw chicken meat, as well as to study the genetic relationship between strains and virulence profiles.**

Methods Nine *Salmonella enterica* strains (5 *S.* Heidelberg; 2 *S.* Enteritidis; 1 *S.* Typhimurium and 1 *S.* Meleagridis) recovered from raw chicken meat marketed in the urban area of Mérida, Venezuela, were studied. Phenotypic characterization was based on antimicrobial susceptibility testing as well as detection of extended-spectrum β-lactamases (ESBLs) by double-disc synergy. The susceptibility to biocides was determined using the dilution-neutralization methods. The detection of quinolone resistance-determining regions of *gyrA, gyrB*, and *parC* genes, bla_{ESBLs} genes, plasmid-mediated quinolone resistance determinants and virulence genes (*invA* and *spvC*) was carried out by PCR. All strains were typed using PFGE.

Results Multidrug-resistance was evident in 6 of 9 strains studied. However, all *Salmonella* serotypes were susceptible to the tested biocides. Genotypic characterization determined that 5 strains harbored the bla_{CTXM-2}, 4 bla_{TEM-1} and 3 *qnrB19* genes. All strains were positive for the *invA* gene. The *spvC* gene was detected in 4 of them. PFGE grouped *Salmonella* strains into 4 different patterns that represented individual serotypes.

Conclusions This study provides valuable information on antibiotic resistance, biocide susceptibility profiles, virulence gene content and genetic diversity of *Salmonella enterica* serotypes isolated from raw chicken meat marketed in Venezuela, and evidenced a health risk for consumers.

Keywords ***Salmonella,*** **antibiotic resistance, biocides, virulence, raw chicken meat**

Introduction

Salmonella enterica is one of the most important foodborne pathogens worldwide.[1-4] Poultry products are most often involved in human salmonellosis outbreaks, infection occurring though cross-contamination from equipment, utensils and human handling of raw products as well as through direct consumption of undercooked poultry meat.[1,2] Salmonellosis associates high rates of morbidity with major economic and health implications. The worldwide number of human infections per year is estimated to be above 93.8 million cases, leading to 155,000 deaths per year.[3,4] In Latin America, Asia and Africa, the reported incidence rate of salmonellosis is 200-500 cases per 100,000 person-year.[1-4] Although in Venezuela there is a poor record of cases of salmonellosis, it is estimated that this infection is among the first twenty causes of death.[5,6]

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The virulence of *Salmonella* is due to a combination of genes present in the bacterial chromosome, plasmids and prophages that determine pathogenicity among their serotypes.[7] *Salmonella* pathogenicity islands (SPIs) are also essential for adhesion, invasion, intracellular survival and systemic infection.[7] The *invA* gene in SPI-1 encodes a type 3 secretion system (T3SS) that allows invasion of phagocytic and non-phagocytic cells. This gene is unique to all *Salmonella* serovars and is a recognized marker for rapid genus detection.[7,8] Moreover, operon *SpvRABCD*, which contains five genes and is present in plasmids common in many serotypes, contributes to colonization of deeper tissues and can increase the severity of enteritis as well as lead to persistence in extra-intestinal sites.[9]

Salmonella pathogenicity in humans is coupled with a rising incidence of antibiotic resistance.[10] Multidrug-resistant (MDR) *Salmonella* strains are often of zoonotic origin, acquiring resistance in animals and subsequently being passed on to humans.[1,3,4,10,11] Salmonellosis is a self-limiting infection of the gastrointestinal tract. However, invasive or prolonged infections of immunocompromised and/or elderly patients, make antibiotic treatment mandatory.[1-4] In these instances, fluoroquinolones and cephalosporins are first choice agents;[1,6,10,11] nevertheless, the increase of MDR in *Salmonella* impacts treatment options.[11] Biocides are used in food manufacturing facilities for controlling microbial contamination. In fact, biocides are essential for the production of high-quality safe food. However, the widespread use of biocides has led to their dissemination into the environment and can lead to bacterial adaptation, and potentially low-level susceptibility to antibacterials, since exposure to biocides can result in cross-resistance to antibacterial agents.[12]

Antibiotic resistant foodborne infections have recently increased in incidence and are deemed as serious public health issues.[1-6] There are few reports in Venezuela describing the genetic basis of *Salmonella enterica* serotypes pathogenicity and mechanisms of resistance to antimicrobials and biocides.[11,13,14] Therefore, the aim of this study was to characterize antimicrobial resistance and biocide susceptibility in *Salmonella enterica* serotypes isolated from raw chicken meat in Venezuela, as well as the genetic relationship between strains and virulence profiles.

Methods

Salmonella strains

A total of 9 *Salmonella enterica* strains (5 S. Heidelberg; 2 S. Enteritidis; 1 S. Typhimurium and 1 S. Meleagridis) with resistance to extended-spectrum cephalosporins and/or an unusual fluoroquinolone resistance phenotype were studied. These strains were recovered from raw chicken meat marketed in the urban area of Mérida, Venezuela, during 2016 and belonging to the collection of non-typhoidal *Salmonella* strains (NTS) of the Molecular Microbiology Laboratory, Faculty of Pharmacy and Bioanalysis, University of Los Andes, Mérida, Venezuela. All strains were identified by conventional methods and serotyped by the *Salmonella* reference center (Instituto Nacional de Higiene "Rafael Rangel", Caracas, Venezuela).

Phenotypic characterization

Antibiotic susceptibility testing. Resistance patterns were determined by minimum inhibitory concentration (MIC) using the standard agar plate dilution method, in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines.[15] The antimicrobial agents tested included (Oxoid Ltd., Basingstoke, UK): ampicillin, cefotaxime, ceftazidime, aztreonam, imipenem, meropenem, ertapenem, nalidixic acid, ciprofloxacin, amikacin, gentamicin, tobramycin, tetracycline and sulfamethoxazole-trimethoprim. *E. coli* ATCC 25922 was used for quality control purposes. The extended-spectrum β-lactamases (ESBL) phenotype was detected by the double-disc synergy test (DDST) according to CLSI guidelines.[15]

Biocide susceptibility testing. Susceptibility of *Salmonella* strains to biocides was determined using the dilution-neutralization method according to the standardized technique by the French Association for Standardization

(AFNOR)[16] and the Spanish Association for Standardization and Certification (AENOR).[17] The biocides evaluated were: lauryl-dimethyl benzylammonium bromide 0.16% v/v (Gerdex: Rodeneza, CA, Caracas, Venezuela), sodium hypochlorite 5% v/v (commercial use) and acetic acid 5% v/v (domestic use). The ranges of dilutions evaluated were: 1:20 (50,000 ppm) and 1:50 (20,000 ppm). The neutralizer used was composed of Tween 80 (30 g/L) and asolectin (3 g/L) (both from Sigma-Aldrich, St Louis, MO, USA). Biocides and the neutralizer were sterilized by autoclaving at 121°C for 15 min at 100 kPa (15 psi). Tests were carried out in Letheen broth (Sigma-Aldrich) from a standardized initial inoculum of 1×10^8 CFU/mL. The strain of S. Typhi ULALMM-8 was used to control bacterial viability. Results were expressed as the reduction factor (RF) when comparing the log number of colony forming units (CFU) before (CFU_0) and after the contact time (CFUc) as follows: RF = $\log_{10}CFU_0 - \log_{10}CFUc$. Bacterial inhibition or biocidal effect was considered positive when the initial CFU/mL decreased by 5 logarithmic units (10^5) in 5 minutes of contact with the disinfectant at 20°C.

Genotypic characterization

Detection of virulence genes. Salmonella strains were analyzed by PCR to detect the presence of virulence genes *inv*A and *spv*C using a previously described method and specific primers.[18]

Detection of mutations in the topoisomerase genes gyrA, gyrB, and parC and plasmid-mediated quinolone resistance (PMQR). PCR was used to amplify the quinolone resistance-determining regions (QRDR) of target genes to identify mutations in each isolate. DNA was prepared and the QRDR of *gyrA*, *gyrB* and *parC* were amplified with previously described primers and protocols.[19] Screening for *qnrA*, *qnrB*, *qnrS*, *qnrD*, *aac(6´)-Ib*, and *qepA* genes was carried out by multiplex and simplex PCR amplifications using a previously described method and specific primers.[11]

Detection of β-lactamase genes. bla_{TEM}, bla_{SHV}, and group bla_{CTX-M} genes were detected by PCR using previously described primers and protocols.[20]

Sequence analysis. All amplification products were purified (PCR-Accu-prep Kit Bioneer, Daejeon, Korea) and their nucleotide sequencing was performed with the 3730XL genetic analyzer (Applied Biosystems, CA, USA). DNA sequences were analyzed using the Basic Local Alignment Search Tool (BLAST) suite of programs (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and compared with those included in the GenBank database in order to determine the specific type of gene.

PFGE genotyping. The clonal relationship of the isolates was determined using the pulsed-field gel electrophoresis (PFGE) technique. Slices of the prepared PFGE plugs were incubated with *Xba*I (Promega, Madison, WI, USA) at a concentration of 20 U/plug for 3 h at 37°C. Plug slices were then inserted into the wells of 1% Seakem Gold Agarose gels in 0.5X TBE buffer (Tris-borate-EDTA) with a CHEF-DR III apparatus (Bio-Rad, Hercules, CA, USA) at 14°C and 6 V/cm for 19.5 h by using pulse times intervals from 2.2 to 54.2 seconds and a 120° switch angle. Bacteriophage lambda concatemers (Bio-Rad) were used as DNA size markers. *Salmonella* Typhimurium ATCC 19585 was used as a positive control.

Results

Table 1 shows the resistance patterns of the *Salmonella* strains to the antibiotics tested, as well as the results of the biocide susceptibility tests. All *Salmonella* strains in this study were resistant to at least two of the 14 antimicrobials tested, showing different resistance patterns. Six strains were resistant to ≥3 different antimicrobial groups, and were considered as multidrug-resistant. Two S. Heidelberg strains (LMM175 and LMM179) were resistant to ≥7 antimicrobials, including aminoglycosides.

Table 1. Phenotypic characterization of *S. enterica* serotypes isolated from raw chicken meat

Salmonella enterica Serotypes (Strain Nº)	Resistance patterns	DDST	CIP (µg/mL)	Dilutions of biocides Reduction factor in log_{10}*					
				NaClO		LDBAB		CH_3COOH	
				1/20	1/50	1/20	1/50	1/20	1/50
Heidelberg (LMM-105)	AMP, CTX, TE, T/S	+	0.25	6.67	6.21	6.20	5.94	5.93	4.95
Heidelberg (LMM-175)	AMP, CTX, CAZ, AZT, TE, T/S, GEN, CIP	+	2	6.42	6.20	6.18	5.49	6.24	5.12
Heidelberg (LMM-179)	AMP, CTX, CAZ, AZT, TE, T/S, GEN, TOB, CIP	+	2	6.73	6.10	6.42	5.47	6.02	4.94
Heidelberg (LMM-182)	AMP, CTX, T/S	+	0.25	5.93	5.39	5.76	5.69	6.08	4.95
Heidelberg (LMM-288)	AMP, CTX, AZT, TE, T/S	+	0.125	6.30	5.97	5.69	5.02	5.67	4.39
Enteritidis (LMM-170)	AMP, TE, T/S	-	0.5	6.30	6.11	5.91	5.23	5.68	5.02
Enteritidis (LMM-218)	AMP, T/S	-	0.5	6.24	6.08	6.19	5.80	5.88	4.91
Typhimurium (LMM-120)	AMP, TE	-	0.25	5.96	5.32	5.84	5.14	5.77	4.78
Meleagridis (LMM-300)	TE, T/S, CIP	ND	2	6.21	5.92	5.95	5.23	5.83	4.81

AMP – ampicillin; **AZT** – aztreonam; **CAZ** – ceftazidime; **CH_3COOH** – acetic acid; **CTX** – cefotaxime; **CIP** - ciprofloxacin; **DDST** – extended-spectrum β-lactamase, double disk synergy test; **GEN** – gentamicin; **LDBAB** – laury dimethyl benzyl ammonium bromide; **NaClO** – sodium hypochlorite; **ND** – not determined; **TE** – tetracycline; **TOB** - tobramycin; **T/S** – trimethoprim-sulfamethoxazole.

*Results are expressed as the reduction factor (RF) when comparing the log number of colony forming units (CFU before (CFU_0) and after the contact time (CFUc) as follows: RF = $log_{10}CFU_0$ – $log_{10}CFUc$

All strains were resistant to ampicillin and trimethoprim-sulfamethoxazole, except for *S.* Meleagridis and *S.* Typhimurium, respectively. Only five of the *Salmonella* strains had a positive ESBL-phenotype. Three strains (LMM175, LMM179 and LMM300) showed decreased susceptibility to ciprofloxacin (2-4 µg/mL). All strains remained fully susceptible to carbapenems, amikacin and nalidixic acid (data not shown). The inhibitory effect of three disinfectants, sodium hypochlorite (NaClO), lauryl-dimethyl-benzyl ammonium bromide and acetic acid (CH_3COOH) was evaluated. Although all the strains' CFU/mL was substantially reduced by two or more orders of magnitude for each disinfectant in the two dilutions evaluated (1:20 and 1:50) during ≤5 minutes, the acetic acid showed a greater inhibitory power on the strains analyzed (4.78-5.12).

The genotypic characterization of *S. enterica* serotypes isolated from raw chicken meat is shown in Table 2. To confirm *Salmonella* strains and prove their virulence, the prevalence of invasion (*invA*) and virulence (*spv*C) genes was determined. All *Salmonella* strains showed a 244 bp DNA fragment for the *Salmonella*-specific *invA* gene, while the *spv*C gene (571 bp) was detected in 4 strains. PCR amplification using specific primers for bla_{TEM}, bla_{SHV}, group bla_{CTX-M} and group *qnr* and sequencing analysis, allowed us to identify $bla_{CTX-M-2}$ in all *S.* Heidelberg and co-existence of bla_{TEM-1} and *qnrB19* in 4 and 2 strains, respectively. The $bla_{CTX-M-2}$ and *qnrB19* were found without association with other resistance markers in the strains *S.* Heidelberg LMM182 and *S.* Meleagridis LMM300, respectively. No mutations were identified in the QRDR of the *gyrA*, *gyrB*, and *parC* genes.

Table 2. Genotypic characterization of *S. enterica* serotypes isolated from raw chicken meat

Strain number	Serotypes	Virulence genes	Resistance genes	PFGE type
LMM105	S. Heidelberg	*invA*, *spvC*	bla_{TEM-1} + $bla_{CTX-M-2}$	A
LMM175	S. Heidelberg	*invA*	bla_{TEM-1} + $bla_{CTX-M-2}$ + *qnrB19*	A
LMM179	S. Heidelberg	*invA*, *spvC*	bla_{TEM-1} + $bla_{CTX-M-2}$ + *qnrB19*	A
LMM182	S. Heidelberg	*invA*, *spvC*	bla_{CTXM-2}	A
LMM288	S. Heidelberg	*invA*	bla_{TEM-1} + $bla_{CTX-M-2}$	A
LMM170	S. Enteritidis	*invA*	-	B
LMM218	S. Enteritidis	*invA*	-	B
LMM120	S. Typhimurium	*invA*, *spvC*	-	C
LMM300	S. Meleagridis	*invA*	*qnrB19*	D

PFGE – pulsed field gel electrophoresis.

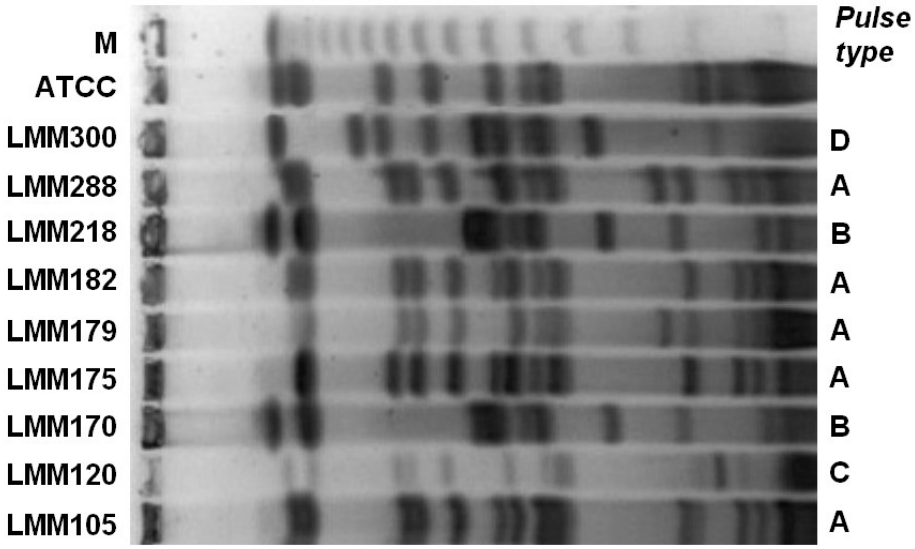


Figure 1. Pulsed field gel electrophoresis (PFGE) types for *Xba*I-digested genomic DNA of *Salmonella enterica* strains isolated from raw chicken meat

Four different PFGE types (**A, B, C, D**) were generated according to serotypes. **M** – λ ladder marker for PFGE; **ATCC** – *Salmonella* Typhimurium ATCC 19585 control strain; **LMM300** – *S.* Meleagridis; **LMM288** – *S.* Heidelberg; **LMM218** – *S.* Enteritidis; **LMM182** – *S.* Heidelberg; **LMM179** – *S.* Heidelberg; **LMM175** – *S.* Heidelberg; **LMM170** – *S.* Enteritidis; **LMM120** – *S.* Typhimurium; **LMM105** – *S.* Heidelberg.

PFGE grouped the 9 *Salmonella* strains into four distinct patterns, each representing one of the following four serotypes: Heidelberg, Enteritidis, Typhimurium and Meleagridis (Figure 1 and Table 2). Pulse types: A grouped 5 S. Heidelberg strains, B grouped 2 S. Enteritidis strains (LMM170 and LMM218) and C and D grouped S. Typhimurium and S. Meleagridis, respectively.

Discussion

The increased antimicrobial resistance in *Salmonella* from retail meats has become a common problem worldwide.[1,4] In this study,

multiple-drug resistance was observed in most of the *Salmonella* strains (6/9). Usually, MDR *Salmonella* are of zoonotic origin.[1,8,10] The increase of antimicrobial resistance among foodborne pathogens can be due to the selection pressure created by either inappropriate or uncontrolled antibiotic use in veterinary medicine,[2,4] as well as to the unregulated antibiotic use in developing countries such as Venezuela.

In this study, based on the patterns of resistance to extended-spectrum cephalosporins and/or resistance to ciprofloxacin with associated susceptibility to nalidixic acid, the presence of ESBL-type and *qnr* genes was suggested in the *Salmonella* strains. Molecular characterization revealed that all S. Heidelberg strains harbored the $bla_{CTX\text{-}M\text{-}2}$ gene and 4 of these were associated to other β-lactamases TEM-1 type (strains: LMM105, LMM175, LMM179 and LMM288). Additionally, in 2 of these strains and S. Meleagridis, the *qnrB19* gene was detected as the only determinant responsible for quinolone resistance. Diverse studies reported the prevalence of *qnr* genes in *Salmonella* isolates from South American countries, being the *qnrB* alleles most frequently detected in Enterobacteriaceae,[3,4,6] in accordance with our results. There are no particular phenotypic traits that allow clinical laboratories to easily recognize the presence of *qnr*-harboring plasmids or others transferable quinolone resistance determinants.[11-14] Clinical isolates positive for plasmid-mediated quinolone resistance (PMQR) determinants did not show any significant MIC change compared to the isolates that were susceptible to nalidixic acid and negative for the presence of *qnr* genes. It is therefore essential to revise the testing practice for susceptibility to fluoroquinolone, to avoid under-detection by clinical laboratories that use traditional phenotypic methods. Furthermore, although *qnr* by itself is only responsible for low-level resistance, it can enable the selection of higher-level resistance mutations. The occurrence of ESBL associated to PMQR in *Salmonella* has been reported worldwide.[1,21,22] Selective pressure exerted by fluoroquinolones may lead to the emergence of isolates positive not only for PMQR determinants but also for ESBL. In 2013, we reported the association of the transferable quinolone-resistance determinant *qnrB19* with ESBL in *Salmonella* strains in Venezuela.[11] However, to date, there is very limited data on the susceptibility to disinfectants in *Salmonella* and their correlation with antibacterial resistance and virulence genes.[12,21] Disinfectants have been extensively used for centuries in many environments including homes, hospitals, farms and food industry as decontaminants. In addition, they have been used on surfaces following slaughter to prevent the dissemination of microorganism and cross-contamination from foodborne pathogens.[23] Hence, the ability to evaluate the efficacy of biocides or disinfectants on *Salmonella* is very important. In this study, all *Salmonella* strains were susceptible to the tested biocides (sodium hypochlorite, lauryl dimethyl benzyl ammonium bromide and acetic acid) used at the manufacturer's recommended rate of dilution (in this context, it is important to take into account the use of biocides at appropriate concentrations). Antibiotic-resistant bacteria, when compared to antibiotic-sensitive strains, have been reported as not significantly more resistant to antiseptics and disinfectants,[21,23] as antimicrobial resistance occurs through alteration of a target that is not also involved in resistance to disinfectants. Conversely, disinfectants can select for antibiotic resistance, as field literature has shown.[12] Therefore, under the pressure of concomitant use of antibiotics and disinfectants, the potential co-selection of resistance genes and the spread of acquired resistance are enhanced.[24] Thus, enforcement of correct disinfection regimens and appropriate usage of antibiotics would still be essential when MDR *Salmonella,* carriers of virulence and pathogenicity genes, are recurrently isolated from food.

Salmonella virulence is due to a combination of chromosomal and plasmid factors.[9] In this study, all strains were confirmed as *Salmonella* by the presence of *invA* gene, a chromosomally located gene encoding a protein responsible for invasion of host epithelial cells, and for allowing the bacteria to survive in macrophages.[7] The presence of the *invA* gene is frequently associated with other virulence genes, which contributes to the

pathogenicity process.[7-9] Our study showed that 4 *Salmonella* strains were positive for the *spv*C gene. This gene, located in plasmids, increases the growth rate of the microorganisms in host cells and affects their recognition by the host immune system.[9] In addition, the *spv*C gene was detected in some strains expressing resistance to fluoroquinolones and/or third generation cephalosporins, which is particularly relevant in the clinical context.

Antimicrobial resistance and virulence of *Salmonella* strains are driving factors of systemic infections.[3,8,10] Additionally, *Salmonella* plasmids encode virulence and MDR genes, and can also be easily disseminated.[24] In fact, three *Salmonella enterica* subsp. *enterica* serovar Typhimurium clones emerging in Europe have been reported to harbor MDR plasmids encoding additional virulence factors.[25] Thus, the study of virulence and resistance genes distribution in different *Salmonella* serotypes would contribute to a better understanding of *Salmonella* pathogenicity.

PFGE has been used to determine clonal relatedness and to define epidemiological links between different bacterial strains. In this study, PFGE grouped *Salmonella* strains into 4 distinct serotypes. We identified no association between virulence profiles and/or antibiotypes and PFGE types, suggesting that the sources of contamination of raw chicken meat are not related.

Conclusions

In this study, the presence of *Salmonella* in raw chicken meat marketed in Venezuela, a potential health risk for consumers, is evidenced. These strains were susceptible to biocides tested, but molecular investigation determined the presence of $bla_{\text{CTX-M-2}}$, $bla_{\text{TEM-1}}$ and *qnrB19* genes associated with virulence factors *inv*A and *spv*C. Control of enteropathogens in food processing can be challenging. Biocides are important in eliminating potential infection sources and the results of this study indicate they are still effective against *Salmonella*. Therefore, the use of biocides at the appropriate concentrations, as well as implementing effective and hygienic measures during production, slaughtering, preparation and processing of chicken meat products, as well as antimicrobial controls in the veterinary medicine stewardship, should be undertaken to reduce dissemination of MDR *Salmonella* in the food chain.

Authors' contributions statement: FG carried out laboratory work, analysis of data, literature review and wrote the initial draft. MA designed and supervised the study and contributed to final writing of the paper and its critical review. Both authors have read and approved the final version of the manuscript.

Conflicts of interest: All authors – none to declare.

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