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Original Research Article

CHARACTERIZATION OF CLASS 1 INTEGRONS AND SOME ANTIMICROBIAL RESISTANCE GENES IN *Salmonella* SPECIES ISOLATED FROM POULTRY IN EGYPT

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Abstract: *Salmonella enterica* includes serotypes that were implicated as a food borne pathogens crucially affecting public health and the economic organization. This study was directed to isolate and identify of *Salmonella* strains from 222 different species and ages of poultry (broiler, chick, ducks, pigeon, quails) from Kafr El Sheikh governorate. The Salmonella isolation rate was (4.5%) as (0.9%) from apparently healthy, (3.6%) from diseased birds. The outer membrane protein F gene was used as promising tool for detection of Genus *Salmonella*, after that four isolates were identified serologically as two *Salmonella enterica* serovar Enteritidis and two *Salmonella enterica* serovar Typhimurium. The resistance pattern of positive Salmonella isolates showed multidrug resistance phenotypes and *qnrS* for quinolone resistant genes was recorded in one isolate while *bla*TEM for β-lactam resistant isolates, *aacC* for aminoglycosides were recorded in all four *Salmonella* isolates using PCR technique. Also, Class 1 integrons detected with a percentage of (100%) in examined isolates. Sequencing of the class 1 integrons cassettes showed genes encoding resistance specified to streptothricin acetyltransferase (*sat*) gene, aminoglycoside acetyltransferase (*aac3-Id*) and aminoglycoside adenyltransferase (*aadA7*). Class 1 integrons harbored gene encoding domains unfunction protein (*duf*) in one *S. enterica* serovar Typhimurium isolate. This study spotlights the significant role of the drug–resistance genes and Class 1 integrons in *Salmonella* as zoonotically important pathogens of public health importance.

Key words: poultry; *Salmonella*; drug- resistance genes; integrons gene cassettes

Introduction

Salmonella include approximately 2500 serovars. *Salmonella enterica* represents the most of the *Salmonella* serovars and *Salmonella enterica* serovar Enteriditis was the most popular serovar with a zoonotic effect, then *Salmonella enterica* serovar Typhimurium (1). Globally, *Salmonella enterica* subsp. *Enterica* included serotypes that have economically and public health significantly effects (2). The most non-typhoidal salmonellosis (NTS) cases related to consuming of contaminated animal

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origin foods, especially fowl, meat and in some cases vegetables (3). Poultry considered an important reservoir of many zoonotically important pathogens, such as Salmonella, which acted as a prime importance (4).

The pore-forming proteins of Salmonella and other Gram negative bacteria outer membrane (OM) called porins (5). Among OMPs (outer membrane proteins), the outer membrane protein F (*ompF*) and outer membrane protein C (*ompC*) were the most types porins that represented 2% of the total porins, and *ompF* was the most ideal structural and functional characterization porin protein (6). Also, the *ompF* gene was used as a promising tool for detection of Salmonellae where it could discriminate genus Salmonella from other non-Salmonella organisms in clinical samples (7).

Multidrug resistant (MDR) non-typhoidal *Salmonella* (NTS) might be transmitted from the poultry to human through the food series, whilst the antimicrobial resistance (AMR) could be carried among bacteria throughout the resistance genes associated with integrons and another mobile genetic elements as plasmids and transposons (8). Avian Salmonella showed resistance against many antimicrobial groups such as β-lactam, aminoglycosides and quinolones (9).

Salmonella species associated with *qnr* genes were isolated from the poultry field might cause a harmful effect on the public health because these could be transmitted to humans via poultry products or by contact with poultry and could rapidly increase fluoroquinolone resistance in various bacterial species through the transfer of plasmids harboring *qnr* genes. (10). The resistance to aminoglycosides as Gentamicin could confer using the aminoglycoside acetyltransferase (*aac*) genes which were detected in numerous isolates of Salmonella (11).

The class 1 integrons played a character in the presence of AMR in *Salmonella enterica* which might isolate from broilers, meat and hogs products (12). Class 1 integrons, the most communal integron located on Salmonella genomic island 1 (SGI 1), was found in various Salmonella serovars, including S. enterica serovar Typhimurium; *S. enterica* serovar Newport and S. enterica serovar Oslo. (13).

The classes of integrons which might be recovered from GenBank were nine, but the first four categories had been sustained only. Cass 1 integrons was widely distributed among the family Enterobacteriaceae organisms (14). These integrons include two conserved segments (5′ CS and 3′ CS) separated by a variable region that normally comprises one or more gene cassettes. Integrons encompass three important parts: an integrase gene (*IntI1*); an adjacent attachment site (attI1) and a promoter region (PC) (15). An open reading frame (ORFs) where a specific site containing a modular structure called gene cassettes (16). The collection of gene cassettes (up to nearly half a dozen) had related the integrons with MDR (17).

This study highlighted the importance of the strains of Salmonella, which isolated from different species and age poultry as zoonotically important pathogen, some antimicrobial drug resistance genes of Salmonella species and class 1 integrons gene cassettes in this public health importance organism.

Materials and methods

Collection of samples and isolates characterization

In this work, which was conducted from April 2017 to April 2018, a total of 222 apparently healthy (56) and diseased (166) from different species and ages of poultry (Broiler, chick, ducks, pigeon, quails) from the Kafr El Sheikh governorate. The internal organs (liver, spleen, gall bladder, ileocaecal tonsil, yolk sac), cloacal swabs and the pooled samples. Samples were transferred to the laboratory in an ice tank within 2hours for bacteriological isolation and identification (18), then confirmed biochemically by the API 20E system.

Serological identification

Biochemically suspected isolates were serotyped according to Kauffman (19) at Serological unit in Institute of Animal Health Research,Giza, Egypt.

Identification of genus Salmonella using ompF gene

Programming of PCR to amplify *ompF* gene was used as promising tool for detection of genus *Salmonella* was done according to Tatavarthy and Cannons (20) using oligonucleotide primers in Table 1.

Antimicrobial susceptibility

The susceptibility test of samples were done as Finegold and Martin, (21). A total of 11 antimicrobial discs was used for sensitivity (Oxoid) were Amoxicillin–clavulanic acid (AMC), 30 µg; Cefotaxime (CTX), 30 µg; Ceftazidime (CAZ), 30 µg; Chloramphenicol (C), 30 µg; Ciprofloxacin (CIP), 5 µg; Gentamicin (CN), 10 µg; Nalidixic acid (NA),30 µg; Spectinomycin (SH), 10 µg; Colistin (CT), 10 µg; Norfloxacin (NOR), 10 µg and Doxycycline (DO), 30 µg. Interpretation as resistant, moderately susceptible or susceptible as recorded in the Clinical and Laboratory Standards Institute CLSI (22).

Molecular analysis of antimicrobial resistance genes

The DNA extraction was done using QIAamp DNA Mini Kit (Catalogue no. 51304) according to manufactures' guidelines. The primer sequences for detection of *aacC* gene (encoded for aminoglycoside resistance) (23), *qnrS* gene (encoded for quinolones resistance) (24), *blaTEM* gene (encoded for β-lactams resistance) (25) and class 1 integrons gene cassettes (26) (Table 1).

Sequencing screen for class 1 integrons gene cassettes

QIAquick kit. (Qiagen Inc. Valencia, CA): It was used for purification of the PCR product from 1.5 % agarose gels. Applied Biosystems 3130 automated DNA Sequencer *(ABI, 3130, USA)*. Identification similarity of nucleotide and amino acid sequences between *Salmonella* strains and other Enterobacteriacae recorded in GenBank was done using (National Center for Biotechnology Information "NCBI"). Using the BioEdit sequence alignment editor for comparisons of the nucleotide sequences (27). Phylogenetic analysis was done using ClustalW (http://www.ebi.ac.uk/clustalw/).

Results

The incidence of Salmonellae from different samples

The obtained results of *Salmonella* isolation revealed that 10 (4.5%) were positive for *Salmonella* identified biochemically out of 222 examined birds, The isolation rates from chicken, ducks and quails were 8 of 156 samples (5.1%), 1 of 35 samples (3.2%) and 1 of 2 samples (50%), respectively, while could no isolation of *Salmonella* from chick and pigeon samples. The positive biochemically *Salmonella* isolates from different samples represented in 4 out of 100 (4%), 2 out of 51 (3.9%), 2 out of 36 (5.5%), 1 out of 19 (5.2%) and 1 out of 2 (50 %) from cloacal swabs, pooled samples, liver, gall bladder and yolk sac samples respectively, while the isolation from the spleen and ileocecal tonsil samples was negative for Salmonellae (Table 2).

Serotyping of Salmonella isolates

Four isolates from ten biochemically positive suspected *Salmonella* isolates were classified under two different serotypes, including two *Salmonella enterica* serovar Enteritidis were isolated from cloacal swab of chicken and duck and *Salmonella enterica* serovar Typhimurium isolated from the quail yolk sac and chicken liver samples.

Antimicrobial susceptibility

Salmonella isolates showed resistance to Gentamycin, Ciprofloxacin, Doxycycline, Spectinomycin and Colistin with (50%), however, showed sensitive to Ceftazidime with (100%), followed by Cefotaxime by (75%), amoxicillin clavulinic acid, Nalidixic acid, Chloramphenicol and Norofloxacine with (50%) (Table 3). Two non-typhoidal *Salmonella* isolates showed multidrug resistant (MDR) phenotypes to five different antibiotic classes (Table 3).

Detection of genus Salmonella using OmpF gene by PCR

All examined *Salmonella* isolates was positive at 519 bp of *ompF* using the PCR technique with a percentage of (100%) (Fig. 1).

Antimicrobial resistance encoding genes

The phenotypic antimicrobial resistant *Salmonella* isolates was analyzed by PCR technique to key out some resistance coding genes. The positive percentage of *qnrS* gene for quinolone resistant was (25%), where *bla*TEM for β-lactam resistant gene, *aacC* for aminoglycosides resistant gene and Class 1 integrons were (100%) (Fig. 1).

Class 1 integrons sequencing of the variable amplicons showed the gene cassettes containing streptothricin acetyltransferase (*sat*) gene encoding resistance against Streptothricin (an early aminoglycoside) in two *Salmonella* serovars isolated in the current work, but aminoglycoside acetyltransferase (*aac(3)-Id*) and aminoglycoside adenyltransferase (*aadA*7) genes which encoding resistance against Gentamycin and to streptomycin and spectinomycin, respectively in isolate of *S. enterica* serovar Typhimurium only. One *S. enterica* serovar Typhimurium isolate Class 1 integrons harbored gene encoding domains of unknown function protein (*duf*).

Table 1: Oligonucleotide primers used for detection of *ompF* , antimicrobial resistance coding genes (*aacC, qnrS* and *bla*TEM) and class 1 integrons cassettes

Gene	Primer sequence (5'-3')	Length of amplified product	Reference
ompF	Forward- CCTGGCAGCGGTGATCC Reverse- TGGTGTAACCTACGCCATC	519 bp	Tatavarthy and Cannons,(20)
aacC	Forward- GGCGCGATCAACGAATTTATCCGA Reverse- CCATTCGATGCCGAAGGAAACGAT	448 bp	Lynne et al., (23)
qnrS	Forward- ACGACATTCGTCAACTGCAA Reverse- TAAATTGGCACCCTGTAGGC	417 bp	Robicsek et al., (24)
*bla*TEM	Forward- ATCAGCAATAAACCAGC Reverse- CCCCGAAGAACGTTTTC	516 bp	Colom et al., (25)
class 1 integron cassettes	Forward- GGC ATC CAA GCA GCA AG Reverse- AAAG CAG ACT TGA CCT GA	Variable	Sow et al., (26)

Table 2: The incidence of Salmonellae isolated from different organs and identified biochemically

Poultry species	Organs samples							Samples No.	positive samples	
	Cloacal swab	*Pooled samples	liver	Spleen	Gall bladder	Yolk sac	iliocecal tonsil		No.	%
Chicken	67	34	29	7	17	-	2	156	8	5.1%
Chicks	6	5	2	2	-	1	3	19	-	0%
Duck	19	10	4	-	2	-	-	35	1	3.2%
Pigeon	8	2	-	-	-	-	-	10	-	0%
Quails	-	-	1	-	-	1	-	2	1	50%
Total	100 (4%)	51 (3.5%)	36 (5.5%)	9 (0%)	19 (5.2%)	2 (50%)	5 (0%)	222	10	4.5%

*Pooled samples from different organs of poultry submitted to Kafr El Sheikh lab. For examination

Table 3: Antimicrobial resistance patterns, resistance genes and class 1 integron profiles of *Salmonella* serotypes in this study

NO	Serovars (source of isolates)	Resistance pattern	**MDR isolates N (%)	Resistance genes	Integron amplicon size (bp)	Genes cassettes	Accession numbers
1	S. *enterica* serovar Enteritidis (duck)	CT	-	*bla*TEM, *aac*C	+	-	-
2	*S. enterica* serovar Enteritidis (chicken)	CN, DO, C, CT, CIP	+	*bla*TEM, *aac*C	600	*sat*	MK335377
3	*S. enterica* serovar Typhimurium (quail)	SH	-	*bla*TEM, *aac*C	800	*duf* gene	MK359461
4	*S. enterica* serovar Typhimurium (chicken)	CN, NOR, DO, AMC, CIP, SH	+	*bla*TEM, *aacC*, *qnr*S	650	*sat*	MK349001
					1600	*aac3-Id,aadA7*	MK349002
					1800	*aadA7*	MK359462
	Total		2(50%)				

**Multidrug resistant (MDR) *Salmonella* isolates were 2(50%) to five different antibiotic classes

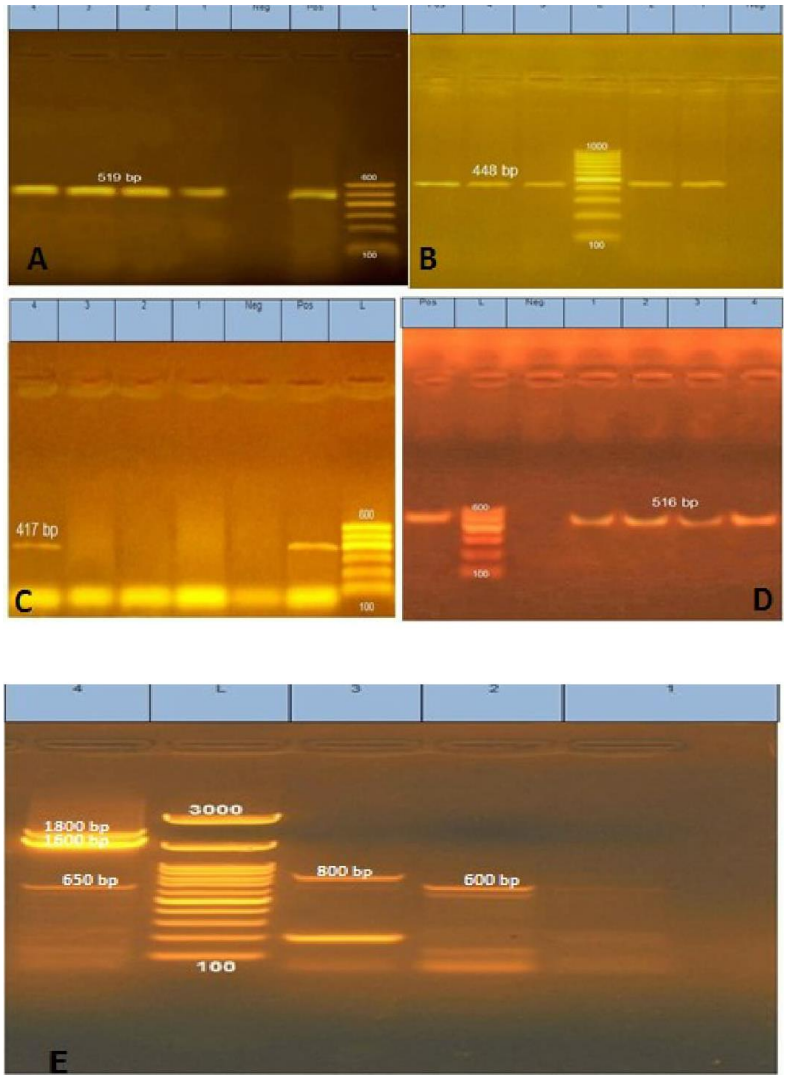

Figure 1: PCR amplification of the different genes in this study; "Pos" stands for positive control, "Neg": Negative control; L: 100 bp DNA ladder; Lane (1, 2, 3, 4) examined *Salmonella* isolates. A. *ompF* gene(519 bp). Resistance associated genes, B. *aacC* gene (448bp). C. *qnr*S gene (417bp). D. *bla*TEM gene (516 bp). E. Class 1 integrons at variable sizes in *Salmonella* isolates

Discussion

Salmonella represents a critical problem to livestock in countries where not interest to the control measures or in those where the environmental conditions help in the development of these microorganisms (28).

In the present study, *Salmonella* spp. were isolated and identified from different species and ages of poultry and molecular characterized for many important antimicrobial resistance genes and class 1 integrons of *Salmonella* species.

The results indicated that 10 (4.5%) isolates out of 222 examined bird suspected to be *Salmonella* isolates from 166 diseased birds and 56 apparently healthy birds with the percentage of (3.6%) and (0.9%), respectively by phenotypic and biochemical characterization that agree with report in Egypt where 4.4% were positive for *Salmonella* isolated from poultry farms (7), but higher than those of *Salmonella* isolation from small poultry farms with (1.6%) in California (29), and lower than (8.65%) of *Salmonella* isolated from poultry (30) .

The consequence of isolation appears to be high from the diseased bird than apparently healthy bird 8vs 2, although the samples were gathered up from each of diseased and apparently healthy birds together. These variations in the overall prevalence of *Salmonella* may be related to several factors such as environment, hygienic conditions of the farm and health status of the examined bird (31, 32) which leading to the bird become weaker and therefore are easily infected by *Salmonella*. Similarly, *Salmonella* was isolated from apparently healthy chickens lower than from diseased chickens in Shanghai and in Egypt (32, 33).

Currently, the isolation percentages from chicken, ducks and quails were 8 of 156 samples (5.1%), 1 of 35 samples (3.2%) and 1 of 2 samples (50%), respectively, were positive for *Salmonella* strains while the chick and pigeon samples were negative for Salmonellae which are not compatible with (7.25%) *Salmonella* incidence from chickens and (15.55%) of pigeons (30) and also with percentage (6%) in ducks in Egypt (34).

The high *Salmonella* isolation rate of liver and gallbladder samples 2 of 36 samples (5.5%), 1 out of 19 samples (5.2%), respectively agrees with the highest rate of *Salmonella* isolation from liver samples (35, 36). All spleen and ceca samples were negative for Salmonellae that agree with another study on *Salmonella* was not isolated from spleen samples (37) but also, disagree with those isolated the highest *Salmonella* percentage from spleen samples in Egypt (38).

It is common knowledge that the cloacal swab is considered a particular signal of incessant intestinal colonization in poultry, but its diagnostic accuracy is minimized where the *Salmonella* infected birds are intermittent shedding via feces (39).

In present the study the four isolated Salmonellae were classified under two different serovars, *Salmonella enterica* serovar Enteritidis and Typhimurium with a percentage of 2 of 4 (50%) for each.

The difference between the results of serological and bacteriological examination to identify *Salmonella* assigned to *Salmonella* give identical colony morphology on S.S agar and biochemical reactions with the other members of the family Enterobacteriaceae and this difference consistent with the opinion of there are problems in the biochemical identification reactions (40). Similarly, there were differences in the identification of *Salmonella* spp. as used conventional techniques was (10.5%), the API 20E system was (9%) and by serotyping was (7.8%) (35).

The serological identification result referred to an isolation of two serotypes, *Salmonella enterica* serovar Typhimurium and Enteritidis, similar that reported in central Ethiopia (41) and Egypt (42) where they isolated only *Salmonella enterica* serovar Enteritidis and Typhimurium, but disagrees with a previous study on *S. enterica* serovar Enteritidis isolated from commercial layer farms (43).

The phenotype antimicrobial resistance result was resistant to (CN), (CIP), (DO), (SH) and (CT) with a percentage (50%). Moreover, the isolates were sensitive to (CAZ) with (100%), followed by (CTX) with (75%) then

(AMC), (NAL), (C) and (NOR) with (50%). These results concur with study reported that the resistance to Gentamycin was observed in (39.58%) (44) and those reported that the resistance to Tetracycline, Ciprofloxacin and Spectinomycin was (51.9%), (48.7%) and (34.4%), respectively (45), but disagree with those reported that Gentamycin inhibited to all *Salmonella* strains and resistance of Ciprofloxacin with a percentage (10%) (46).

The high sensitivity to Ceftazidime (100%), followed by Cefotaxime (75%) in the present work agrees with the previous reports described a low Cephalosporin resistance prevalence of *S. enterica* serovar Enteritidis in Kohat and Egypt (47, 48). Two non-typhoidal *Salmonella* isolates (50%) showed multidrug resistant (MDR) phenotypes to at least five various antibiotic types which similar with another study reported that the multidrug resistant *Salmonella* isolates represented 55% in Malaysia (49).

The outer membrane protein F (*ompF*) gene detected in the examined isolates in this current study with a percentage of (100%) using the PCR technique. The *ompF* gene considers a good tool for fast identification of *Salmonella*, so *ompF* mutation or loss might lead to mistakes in the identification analyze of *Salmonella* strains (20). Similarly, using the *ompF* gene as a tool for detection of *Salmonella* genus in Egyptian poultry farms (7).

Poultry acts as a carrier of multidrug resistant *Salmonella* and this no related to resistance genes presence, so other acquiring resistance mechanisms might be present (50). The detection result of resistance coding genes (*bla*TEM and *aacC*) was (100%) and this disagrees with a previous report detected *bla*TEM in *S. enterica* serovar Typhimurium isolates only in Japan (51), also with another report detected *aacC* gene with (30%) of *Salmonella* isolates in broiler in Egypt (52). The result of the current study similar to another study detected the *bla*TEM gene with (93.3%) in *Salmonella* isolates obtained from commercial layers in Egypt (31). The *qnrS* gene, a gene quinolone resistant was reported in the present work with the percentage of (25%) that parallel with the result of another study in Egypt (31).

The differences in phenotypic-genetic antibiotic resistance results recorded in this study of *Salmonella* isolates was also registered in other reports (53), and was usually mentioned to either existence of resistance alternative mechanisms or defect in the resistant genes expression .

The result of Class 1 integrons detection was (100%) of this work, similarly, the result of Class 1 integrons detection of *Salmonella* isolated from Egypt (54).

The *sat* gene was detected within class 1 integrons of *S. enterica* serovar Enteritidis (chicken, 600bp) and *S. enterica* serovar Typhimurium (chicken, 650bp) in this investigation was preceding identity in *S.* Typhimurium (KT449570) in Egypt (54), *S.* Choleraesuis (EU834941) in southern Taiwan (55), other family Enterobacteriaceae organisms class 1 integrons as, *Shigella sonnei* from western Ireland (AY090896) (56), *E.coli* plasmid (CP022735) (57), and other bacteria as, in *Vibrio alginolyticus* plasmid (KU160531) (58). The *aac (3)-Id* and *aadA7* genes had been identified in class 1 integrons gene cassettes of (*S.* Typhimurium, chicken) showed a preceding identity in class1 integrons of *S.* Derby (KT427378), *S. enterica* (KT581256) in Egypt (54).

In the current investigation, the detection of *sat*, *aac (3)-Id* and *aadA7* genes within class1 integrons of isolated *Salmonella* may be related to the extensive using the aminoglycoside antibiotics group in poultry farms.

The domains of unknown function protein (*duf*) gene was identified in class 1 integrons gene cassettes of (*S.* Typhimurium, quail) in the current work, which difficult to decide its function due to lack of its protein sequences identity with interpreted biochemical function. The *duf* gene represents more than (20%) of all protein domains (59).

The class 1 integrons cassettes sequencing of the two isolated *Salmonella* serovars in this current investigation were documented into the GenBank with accession numbers (MK335377); (MK349001); (MK349002), (MK359462) and (MK MK359461).

Conclusion

Poultry acts as the important reservoir of many zoonotically important pathogens, such as *Salmonella* and detection of resistance genes related to significant antimicrobial drugs which used in the medical establishments. Integrons cassettes carrying antimicrobial resistance genes in *Salmonella* have an important role in the spreading of AMR so, the strategy used to control of using of antimicrobial drugs against this organism as well as other emerging pathogens of public health importance should be improved.

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Conflict of interest

The authors declare that they have no conflict of interest.

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