Original article

Spread of CTX-type extended-spectrum-β-lactamase-producing *Escherichia coli* isolates of epidemic clone B2-O25-ST131 among dogs and cats in Japan

Kumiko Kawamura[1*], Tae Sugawara[1], Nao Matsuo[1], Kengo Hayashi[1], Chihiro Norizuki[1], Kiyoko Tamai[3], Takaaki Kondo[1], Yoshichika Arakawa[2]

[1]Department of Pathophysiological Laboratory Sciences; [2]Department of Bacteriology, Nagoya University Graduate School of Medicine, Nagoya, Aichi, Japan, [3]Miroku Medical Laboratory, Co., Ltd., Saku, Nagano, Japan

[1]Department of Pathophysiological Laboratory Sciences, Nagoya University Graduate School of Medicine.
1-1-20, Daikominami, Higashi-ku, Nagoya, Aichi, 461-8673, Japan
[2]Department of Bacteriology, Nagoya University Graduate School of Medicine
65, Tsurumai, Showa-ku, Nagoya, Aichi, 466-8550, Japan
[3]Miroku Medical Laboratory, Co., Ltd.
659-2, Innai, Saku, Nagano, 384-2201, Japan

Running title: Spread of B2-O25-ST131 ESBL producers in pets

*Corresponding author. Tel: +81-52-719-3116; Fax: +81-52-719-1506; E-mail: kumiko@met.nagoya-u.ac.jp

Abstract

This study was performed to investigate the carriage rates of CTX-M-type extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* among ill companion animals in Japan. Among the 178 non-repetitive *E. coli* isolates, including 131 from dogs and 47 from cats, collected between September and November 2015, 42 (23.6%) isolates from 29 dogs and 13 cats were identified as ESBL producers. The antimicrobial susceptibility, O serotype, phylogenetic group, β-lactamase genotype, plasmid replicon type, and sequence type (ST) of each isolate were analyzed. The major ESBL types were CTX-M-14 (26.8%), CTX-M-15 (24.4%), CTX-M-27 (19.5%), and CTX-M-55 (19.5%); predominant replicon types of $bla_{\text{CTX-M}}$-carrying plasmid were IncF group and IncI1-Iγ. The most prevalent STs were ST131 (n = 15, 35.7%), followed by ST38, ST10, and ST410. The 15 isolates of ST131 belonged to B2-O25. *E. coli* B2-O25-ST131 isolates harboring $bla_{\text{CTX-M-15}}$ or $bla_{\text{CTX-M-27}}$ were resistant to ceftazidime and ciprofloxacin. In particular, CTX-M-15 producers showed multidrug resistance. Our results demonstrated that the CTX-M-producing pandemic *E. coli* clone B2-O25-ST131 has already spread in Japanese companion animals as well. Moreover, the similarity of genotypes, serotypes, phylogenetic groups, and STs of the isolates from companion animals to those from humans suggested probable transmission of resistant bacteria between pets and humans.

51 **Introduction**

52 The production of extended-spectrum β-lactamases (ESBLs) is one of the most common
53 acquired cephalosporin resistance mechanisms in Enterobacteriaceae.[1] Since the 2000s, the
54 resistance rate to third-generation cephalosporins, including cefotaxime and ceftriaxone, in
55 *Escherichia coli* isolates has increased rapidly because of the dissemination of isolates
56 producing CTX-M-type enzymes.[2] In particular, a specific *E. coli* lineage that produces
57 CTX-M-15 and belongs to phylogenetic group B2, serotype O25:H4, and sequence type 131
58 (ST131) has spread globally as a pandemic clone.[3] The worldwide spread of
59 B2-O25:H4-ST131 CTX-M-15-producing *E. coli* clone has become a serious public health
60 concern, since this clone generally shows co-resistance to fluoroquinolones (FQs),
61 aminoglycosides, and trimethoprim (TMP)/sulfamethoxazole (SMX).[4]

62 ESBL-producing *E. coli* isolates have been frequently recovered from non-human
63 sources including food-producing and companion animals as well as the environment.[5-9] In
64 particular, the companion animals such as dogs and cats are considered as possible reservoirs
65 of antimicrobial-resistant bacteria, given their close contact with humans and the extensive
66 use of antimicrobial agents approved for humans in companion animals. Indeed, the
67 transmission of resistant bacteria between companion animals and humans has been
68 documented.[10] Of greater concern is that pandemic clone B2-O25:H4-ST131
69 CTX-M-15-producing *E. coli* has also been isolated from companion animals in various
70 European countries.[11]

71 Japan faces a similar situation because broad-spectrum cephalosporins have been
72 widely used for treatment of bacterial infections in veterinary medicine. However, in Japan,
73 data on the carriage rates and characteristics of ESBL-producing *E. coli* in companion

74 animals are limited.[12,13] A recent investigation found that B2-O25-ST131
75 CTX-M-15-producing *E. coli* is increasingly being isolated from human clinical specimens in
76 Japan,[14] leading us to speculate that companion animals may act as a reservoir for these
77 drug-resistant microorganisms also in our country.

78 To address this issue, the present study investigated the prevalence of CTX-M type
79 ESBL-producing *E. coli* isolates among ill companion animals. In particular, we focused on
80 the emergence and spread of isolates of the CTX-M-producing *E. coli* pandemic clone
81 B2-O25-ST131 in order to evaluate the possibility of their transmission between humans and
82 companion animals.

83

84 **Materials and Methods**

85 *Bacterial isolates*

86 A total of 1,167 clinical specimens were collected from ill pets across Japan between
87 September and November 2015, and subjected to microbial examination by a private
88 company for diagnostic service, Miroku Medical Laboratory Co., Ltd. (Saku, Nagano, Japan).
89 Among the total of 1,167 specimens, 1,112 bacterial isolates were recovered from 812
90 specimens because multiple bacterial isolates were obtained from some of these specimens.
91 Finally, a total of 178 non-repetitive *E. coli* isolates were obtained from clinical specimens of
92 131 ill dogs and 47 ill cats. The sources were mainly urine (102 samples), pus (23 samples),
93 and nasal cavity (14 samples); specimens were also acquired from intrauterine liquid (11),
94 otorrhea (8), and other sources (20), including ascites, pleural effusion, and skin. Stool
95 samples were not included.

96 The phenotype of each isolate was confirmed using the VITEK 2 System (Sysmex

bioMérieux, Tokyo, Japan); those showing resistance to extended-spectrum cephalosporins were further evaluated for ESBL production with a phenotypic confirmatory test using both cefotaxime (CTX) and ceftazidime (CAZ) alone and in combination with clavulanic acid according to the Clinical Laboratory Standards Institute (CLSI) guidelines.[15]

Characterization of genes encoding β-lactamase

The presence of CTX-M β-lactamase-encoding genes was detected by PCR amplification.[16] Additional consensus primers CTX-M/F (5′-TTT GCG ATG TGC AGT ACC AGT-3′) and CTX-M/R (5′-CTC CGC TGC CGG TTT TAT C-3′) were also used. After classification into CTX-M-1, -2, -8, and -9 groups, the CTX-M-1 and CTX-M-9 groups were subjected to nucleotide sequence analyses. Sequencing of *bla*$_{\text{CTX-M-1}}$ group genes was performed using original external PCR primers CTXMGp1F (5′-TCG TCT CTT CCA GAA TAA GGA ATC-3′) and CTXMGp1R (5′-GTT TCC CCA TTC CGT TTC CG-3′) and the following cycling conditions: 95°C for 5 min, 30 cycles of 94°C for 30 s, 59°C for 30 s, and 72°C for 60 s, and a final extension at 72°C for 7 min, which yielded a 925-bp amplicon. Sequencing analyses of *bla*$_{\text{CTX-M-9}}$ group genes was performed as previously described[17] using primers CTXGp9F (5′-GAT TGA CCG TAT TGG GAG TTT G-3′) and CTXMGp9R (5′-ATT TAC TTC CAT TAC TTT GCG G-3′), yielding a 1,086-bp amplicon. The *bla*$_{\text{TEM}}$ and *bla*$_{\text{SHV}}$ genes were PCR-amplified and the entire genes of positive isolates were sequenced as previously described.[18] The six major plasmid-mediated AmpC (pAmpC) genes were detected by PCR amplification and sequenced as previously described.[19] Sequence analyses and comparisons to known sequences were performed with the BLAST programs on the National Center for Biotechnology Information website

(http://www.ncbi.nlm.nih/gov/BLAST).

Transformation and plasmid replicon typing

Plasmid DNA was prepared using the PureYield Plasmid Midiprep System (Promega, Madison, WI, USA) according to the manufacturer's instructions, then transformed into *E. coli* DH10B cells by electroporation using a Gene Pulser Xcell system (Bio-Rad, Hercules, CA, USA).[20] Transformants were selected on Müller-Hinton agar (Becton Dickinson, Sparks, MD, USA) plates supplemented with 1 mg/L CTX (Wako Pure Chemical Industries, Osaka, Japan), and their acquisition of $bla_{\text{CTX-M}}$ was confirmed by PCR. The plasmid replicon types of the resultant transformants were determined by PCR-based replicon typing using 18 primer pairs.[21]

Phylogenetic grouping, multilocus sequence typing (MLST), and H30/H30-Rx subclonal classification within ST131

The phylogenetic group of ESBL-producing *E. coli* isolates was determined by multiplex PCR as previously described.[22] Multilocus sequence typing (MLST) was performed by analyzing seven housekeeping genes (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, and *recA*) (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli). In addition, ST131 isolates were assessed for *H30* status by subclone-specific PCR.[23] Ciprofloxacin-non-susceptible *H30* isolates were classified as *H30*R,[24] and further assessed for *H30*Rx status by subclone-specific PCR.[25]

O serotyping of E. coli and detection of serotype O25b by PCR

Serotype O25 was identified using *E. coli* antisera (Denka Seiken, Tokyo, Japan) according to

the manufacturer's instructions. Genetic O25b serotyping was also confirmed by PCR.[26]

Antimicrobial susceptibility testing

CTX, CAZ, imipenem (IPM), gentamicin (GEN), kanamycin (KAN), amikacin (AMK), fosfomycin (FOM), chloramphenicol, tetracycline (TET), ciprofloxacin (CIP), TMP, and nitrofurantoin (NFT) were obtained from Wako Pure Chemical Industries; SMX was from Merck & Co. (Kenilworth, NJ, USA). SMX and TMP were used at a 5:1 ratio. Antimicrobial susceptibility was determined using the agar dilution method in accordance with CLSI guidelines,[15] and minimum inhibitory concentrations were interpreted according to CLSI criteria (document M7-A10).[27] *E. coli* ATCC25922 was used as the control strain.

Detection of plasmid-mediated quinolone resistance (PMQR) gene by PCR

The PMQR genes *qnrA, qnrB, qnrS, qepA, aac(6′)-Ib-cr,* and *oqxAB* were detected by PCR as previously described.[28-30]

Statistical analysis

Data were analyzed using statistical analysis software "R version 3.3.1". Chi-square tests for the distribution of sequence types, plasmid replicon types, and phylogenetic groups among different $bla_{\text{CTX-M}}$-harboring isolates or among different sequence types were performed. The Benjamini-Hochberg procedure was used to correct multiple comparison *p*-values.[31] A Cochran-Mantel-Haenszel chi-square test was performed to evaluate the dissimilarity in the distribution of antibiotic-resistant isolates between serotypes O25 and non-O25.[32] *P*-values of < 0.05 indicated statistical significance.

166

167 **Results**

168 *Prevalence of ESBL-producing E. coli isolates*

169 The production of ESBLs in 178 *E. coli* isolates was evaluated with a phenotypic
170 confirmation test. A total of 42 (23.6%) ESBL-positive and distinct *E. coli* isolates were
171 obtained from sick dogs (n = 29) and cats (n = 13). Many isolates were recovered from
172 animals that had urinary tract infections (UTIs); 25 (59.5%) were obtained from urine
173 specimens, while the remaining isolates were obtained from various specimens including
174 those from the skin, pus, and nasal cavity (Supplemental Table 1).

175

176 *Detection of genes encoding ESBLs and pAmpC*

177 Of 42 ESBL-producing *E. coli* isolates, 41 harbored the $bla_{\text{CTX-M}}$ gene and one had the
178 $bla_{\text{SHV-12}}$ gene. There were eight variants of $bla_{\text{CTX-M}}$, including $bla_{\text{CTX-M-14}}$ (11/41, 26.8%),
179 $bla_{\text{CTX-M-15}}$ (10/41, 24.4%), $bla_{\text{CTX-M-27}}$ (8/41, 19.5%), and $bla_{\text{CTX-M-55}}$ (8/41, 19.5%) (Table
180 1). One ESBL producer isolated from a dog urine specimen harbored $bla_{\text{CTX-M-123}}$, which
181 encodes a hybrid β-lactamase of CTX-M-15 and CTX-M-14. Among the 42 ESBL-producing
182 *E. coli* isolates, 19 isolates harboring any of the ESBL genes ($bla_{\text{CTX-15}}$, n = 3; $bla_{\text{CTX-M-55}}$, n
183 = 5; $bla_{\text{CTX-2}}$, n = 1; $bla_{\text{CTX-M-8}}$, n = 1; $bla_{\text{CTX-M-14}}$, n = 6; $bla_{\text{CTX-M-27}}$, n = 1; $bla_{\text{CTX-M-123}}$, n =
184 1; and $bla_{\text{SHV-12}}$, n = 1) also co-harbored the $bla_{\text{TEM-1}}$ gene, while no isolates harbored
185 pAmpC genes.

186

187 *Relationships among STs, ESBL genes, and plasmid replicon types*

188 MLST analysis revealed 16 STs, including ST131 (n = 15, 35.7%), ST38 (n = 5, 11.9%),

ST10, ST70, ST162, and ST410 (n = 3 each, 7.1%) (Table 1). All 15 isolates belonging to ST131 were identified as *H30* by *fimH*-based subclonal typing. All ST131-*H30* isolates were ciprofloxacin-resistant, and, therefore, were classified as *H30*R. Among them, there were five *H30*Rx isolates as evidenced by subclone-specific PCR. With respect to relationships between STs and ESBL genes, the STs of the isolates harboring $bla_{\text{CTX-M-14}}$, $bla_{\text{CTX-M-15}}$, or $bla_{\text{CTX-M-55}}$ belonged to different STs, including ST10, ST38, ST70, ST131, and ST410 (Table 1). Although all 8 isolates harboring $bla_{\text{CTX-M-27}}$ were ST131, there was no statistically significant difference in the distribution of ST131 between different ESBL genotypes as evidenced by multiple comparisons with other STs.

Transformation of *E. coli* DH10B with plasmids that mediate CTX resistance was successful for 31 (73.8%) of the 42 donor isolates. The replicon types of the transformed plasmids were IncF group (n = 14, 33.3%) and IncI1-Iγ (n = 11, 26.2%), whereas those of the remaining six plasmids were undetermined by PCR-based replicon typing using 18 primer pairs. Of the 15 ESBL producers assigned to ST131, 10 harbored the plasmid of replicon types of IncF group (Table 1). ST131 had a tendency of a slight distribution difference compared with other STs ($p = 0.0735$), although it was not statistically significant.

Among the eight plasmids harboring $bla_{\text{CTX-M-55}}$, five were of replicon type IncI1-Iγ, while 8 plasmids harboring $bla_{\text{CTX-M-27}}$ showed multiple replicon types of the IncF group. Eleven plasmids harboring $bla_{\text{CTX-M-14}}$ showed diverse replicon types, including IncI1-Iγ and multiple replicon types of the IncF group such as IncFIA, FIB, FII and IncFIA, FII (Table 2). Among different CTX-M types, statistically significant difference was observed in the distribution of replicon types of the plasmids harboring $bla_{\text{CTX-M-27}}$ ($p < 0.05$).

212 *Link between phylogenetic groups and STs*

213 The 42 ESBL-producing *E. coli* isolates were divided into seven phylogenetic groups,
214 including group B2 (n = 16, 38.1%), group D (n = 9, 21.5%), and group A (n = 5, 11.9%)
215 (Table 3). Of the 25 isolates belonging to groups B2 and D, 16 were obtained from urine
216 specimens (group B2, n = 10; group D, n = 6) (Supplemental Table 1). Some STs and
217 phylogenetic groups were linked, such as ST131 and group B2, ST10 and group A, ST162
218 and group B1, and ST38 and group D (Table 3). Phylogenetic group B2 had a statistically
219 significant link to ST131 ($p < 0.01$), while group D was significantly associated with ST38 (p
220 < 0.05).

221 Serotype O25 was established using *E. coli* antisera and by PCR. Among the 42 ESBL
222 producers, 14 were determined as O25b by PCR and one isolate was O25 based on the
223 antiserum. All these 15 isolates belonged to phylogenetic group B2-ST131.

224

225 *Antimicrobial susceptibility profiles*

226 ESBL-producing *E. coli* isolated from clinical specimens of ill dogs and cats were susceptible
227 to IPM, AMK, FOM, and NFT (Table 4). However, these isolates showed high resistance to
228 CTX (100%) and CIP (88.1%), and tended to be resistant to CAZ, GEN, KAN, TET, and
229 TMP/SMX. In addition, most isolates showed a multidrug-resistance phenotype, with 23/42
230 (54.8%) resistant to more than four antimicrobial agents (Supplemental Table 1). All the
231 serotype O25 isolates harboring $bla_{\text{CTX-M-15}}$ (n = 6), $bla_{\text{CTX-M-14}}$ (n = 2), and $bla_{\text{CTX-M-27}}$ (n =
232 7) were resistant to CIP (Table 4). Serotype O25 isolates harboring $bla_{\text{CTX-M-15}}$ exhibited a
233 multidrug-resistance phenotype, whereas those harboring $bla_{\text{CTX-M-27}}$ were susceptible to
234 GEN, KAN, and TET. The distribution of drug-resistant isolates between O25 and non-O25

serotypes was analyzed using the Cochran-Mantel-Haenszel chi-square test, indicating a statistically significant difference between the two serotypes ($p < 0.01$).

PCR screening for detection of PMQR genes revealed the presence of the *aac(6')-Ib-cr* gene in seven isolates harboring $bla_{\text{CTX-M-15}}$ (ST131, n = 5; ST410, n = 2) and one harboring $bla_{\text{CTX-M-14}}$ (ST10). In addition, the isolate harboring $bla_{\text{CTX-M-123}}$ was positive for the *qnrS* gene.

Discussion

Most studies of bacterial infections in animals have focused on *Salmonella* and *Campylobacter* species.[10] However, the relationship between companion animals and their owners has changed substantially over the years in Japan and other countries. Specifically, cats and dogs are regarded as actual family members, leading to a high frequency of close physical contact with humans, which may increase the possibility for transmission of antimicrobial-resistant bacteria in community settings. In Japan, CTX-M-15-producing *E. coli* isolates of pandemic clone B2-O25-ST131 are increasingly detected in hospitals and other settings.[14] This study investigated the role of companion animals as carriers of drug-resistant *E. coli* to address the reason for the wide dissemination of pandemic *E. coli* clones in the community.

The rate of isolation of ESBL producers from clinical specimens of sick companion animals was 23.6%, which is significantly higher than those in many countries,[8] although much lower than that in China (40.4%).[33] This finding may reflect the general trend of using antimicrobials in ill companion animals in Japan. Common use of antimicrobials in pets may account for colonization of ESBL-producing *E. coli* as a result of drug-resistance *E. coli*

transmission between companion animals and their owners.

The differences in the molecular types of ESBLs have been documented in many reports from various countries and regions; thus, CTX-M-14 and CTX-M-55 are mostly found in China,[33] while CTX-M-1 and CTX-M-15 are observed in Italy and the USA.[34,35] In the present study, CTX-M-14-, CTX-M-15-, CTX-M-27-, and CTX-M-55-type β-lactamase producers were detected in almost equal rates. It was previously reported in Japan that CTX-M-27-producing *E. coli* isolates were predominant, whereas CTX-M-15-type β-lactamase strains were rarely found in companion animals.[12] Here, we detected B2-O25-ST131 *E. coli* isolates producing CTX-M-15 (11.9%), CTX-M-14 (2.4%), and CTX-M-27 (19.0%) types of β-lactamases; these results suggest that CTX-M-15-producing B2-O25-ST131 clone has been spreading among companion animals in Japan for several years. Many animals tested in this study had UTIs. *E. coli* isolates belonging to serotype O25 can readily adhere to human and animal bowels,[36] causing UTIs. It was recently reported that O25 was the most frequent O serotype among ESBL producers (CTX-M-14 and CTX-M-27 types) isolated from fecal specimens of healthy Japanese individuals.[37] In the present study, genetic types, serotypes, phylogenetic groups, and STs were similar between humans and companion animals, suggesting possible transmission of ESBL-producing *E. coli* between them. Overall, our findings together with previous data suggest that the spread of the ESBL-producing pandemic clone must be more closely monitored in both humans and companion animals.

The predominant replicon types observed in this study were IncI1-Iγ and the IncF group. The plasmid carrying $bla_{\text{CTX-M-27}}$ had multiple replicon types of the IncF group, whereas that carrying $bla_{\text{CTX-M-15}}$ had the IncF group and IncI1-Iγ types, which is consistent

281 with earlier reports.[38,39] It was previously found that STs and Inc types existed in different
282 combinations; thus, a human ST131 isolate harbored a plasmid carrying multiple replication
283 origins of the IncF group, while in animal ST10, ST58 and ST117 isolates, IncI1-carrying
284 plasmids were detected.[39] These findings are consisted with our result that ST131 was
285 associated with plasmids carrying multiple replication origins belonging to the IncF group,
286 although combinations between ST and Inc type differed in the other STs. Nonetheless, the
287 results of the present study demonstrate that *E. coli* isolates from companion animals are very
288 similar to those from humans.

289 Phylogenetic lineages of antimicrobial resistance and virulence in ESBL-producing *E.*
290 *coli* have been widely studied. ST131 isolates have disseminated to various animal species,
291 including poultry, pigs, and companion animals, as well as humans, and their ST was found
292 to be closely linked to phylogenetic group B2.[40,41] Moreover, the association between
293 different STs and phylogenetic groups, e.g., group D (STC38, ST405, and STC69), A (ST10,
294 ST167, and ST617), and B1 (ST410), has been identified.[8] Our data revealed similar links
295 between STs and phylogenetic groups, confirming that CTX-M-producing *E. coli* B2-ST131
296 isolates are predominant in companion animals. Moreover, we identified CIP-resistant
297 D-ST405, which has been reported as the second most prevalent ESBL-producing clonal
298 group among clinical isolates in Japan.[14] These results would suggest a probable transmission
299 of ESBL-producing *E. coli* isolates between humans and companion animals.

300 Of greater concern is the fact that ESBL producers are frequently resistant not only to
301 β-lactams but also to FQs, TMP/SMX, and aminoglycosides. Our results showed that
302 resistance rates to these three agents were > 35%, whereas that to CIP was remarkably high
303 (88.1%). The proportion of ESBL-producing *E. coli* isolates resistant to FQs appeared to

have increased in parallel with plasmid-mediated resistance mechanisms such as Qnr proteins (*qnrA*, *qnrB*, or *qnrS*), aminoglycoside acetyltransferase variant enzyme [*aac(6')-Ib-cr*], or efflux pumps (*qepA* or *oqxAB*).[42] We found here that CTX-M-15-producing *E. coli* isolates harbored a variant of the *aac(6')-Ib-cr* gene, which is in agreement with a previous study.[11]. PMQR determinants are still rare;[43] however, CTX-M-15-producing *E. coli* ST131 isolates have spread among companion animal populations and may well serve as a reservoir of plasmids conferring resistance to FQs and aminoglycosides. Interestingly, most of the CTX-M-15 and CTX-M-27 producers belonged to serotype O25 and showed multiple resistance profiles. On the other hand, most of the CTX-M-55 and CTX-M-14 producers did not belong to serotype O25, but nevertheless exhibited multiple drug resistance. Thus, differences among pet isolates remain unclear; the relationship between the O serotype and CTX-M type should be clarified in future studies.

In conclusion, we found that CTX-M-producing *E. coli* isolates derived from a pandemic clone B2-O25-ST131 have already widely spread across companion animal populations in Japan. The CTX-M genes, phylogenetic groups, plasmid replicon types, and antimicrobial susceptibility profiles were highly similar to those of human isolates. These results strongly suggest that drug-resistant bacteria are being transmitted between companion animals and humans in Japan, which may be promoted by close physical contact between humans and household pets as well as by the use of the same classes of antimicrobial agents. Continuous studies are required to confirm a potential zoonotic link among ESBL-producing *E. coli* isolates from companion animals and human cases of infections.

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Disclosure statement

All authors have read and approved this article; there are no conflicts of interest to declare.

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