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Phenotypic and genotypic characteristics of *Trueperella pyogenes* isolated from ruminants

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Abstract. *Trueperella pyogenes* is an opportunistic pathogen that causes suppurative infections in animals including humans. Data on phenotypic and genotypic properties of *T. pyogenes* isolated from ruminants, particularly goats and sheep, are lacking. We characterized, by phenotypic and genotypic means, *T. pyogenes* of caprine and ovine origin, and established their phylogenetic relationship with isolates from other ruminants. *T. pyogenes* isolates (n = 50) from diagnostic specimens of bovine (n = 25), caprine (n = 19), and ovine (n = 6) origin were analyzed. Overall, variable biochemical activities were observed among the *T. pyogenes* isolates. The fimbriae-encoding gene, *fimE*, and neuraminidase-encoding gene, *nanH*, were, respectively, more frequently detected in the large (p = 0.0006) and small (p = 0.0001) ruminant isolates. Moreover, genotype V (*plo*/*nanH*/*nanP*/*fimA*/*fimC*) was only detected in the caprine and ovine isolates, whereas genotype IX (*plo*/*nanP*/*fimA*/*fimC*/*fimE*) was solely present in the isolates of bovine origin (p = 0.0223). The 16S rRNA gene sequences of all *T. pyogenes* isolates were clustered with the reference *T. pyogenes* strain ATCC 19411 and displayed a high degree of identity to each other. Our results highlight phenotypic and genotypic diversity among ruminant isolates of *T. pyogenes* and reinforce the importance of characterization of more clinical isolates to better understand the pathogenesis of this bacterium in different animal species.

Key words: Cattle; genotypic properties; goats; sheep; *Trueperella pyogenes.*

Introduction

Trueperella pyogenes (formerly known as *Arcanobacterium pyogenes, Actinomyces pyogenes*, and *Corynebacterium pyogenes*) commonly inhabits skin and mucous membranes of the upper respiratory, gastrointestinal, and urogenital tracts of animals.[7,10,19] In a number of animal species, including wildlife, this gram-positive, opportunistic bacterium causes a variety of suppurative infections including abscesses, arthritis, endocarditis, mastitis, metritis, osteomyelitis, pneumonia, and vasculitis.[2,3] In domestic animals, mastitis (45%), abscesses (18%), pneumonia (11%), and lymphadenitis (9%) are the most common clinical manifestations.[11]

Previous work has genotypically characterized ruminant isolates of *T. pyogenes* recovered from European bison, cattle, and white-tailed deer.[3,6,9,15–17] All reported isolates of *T. pyogenes* carried the pyolysin gene (*plo*),[3,15,17] which encodes an offensin that binds to cholesterol-containing rafts resulting in formation of pores in the host cell membrane followed by apoptosis.[13] Screening for other virulence genes, for example, those coding for neuraminidase P (*nanP*), neuraminidase N (*nanH*), collagen-binding protein (*cbpA*), fimbrial subunits of type A, C, E, and G (*fimA, fimC, fimE*, and *fimG*, respectively), demonstrated that the genotypic profiles varied greatly among *T. pyogenes* isolates.[3,15,17]

Despite earlier studies, data on phenotypic and genotypic characteristics of *T. pyogenes* isolated from cattle, goats, and sheep are still lacking. To date, very few *T. pyogenes* isolates recovered from small ruminants have been analyzed genotypically.[6,14,16] Therefore, we identified the phenotypic and genotypic characteristics of *T. pyogenes* isolates recovered from infections of small and large ruminants and established the phylogenetic relationship of these isolates with previously characterized *T. pyogenes* isolates from other ruminants.

Materials and methods

T. pyogenes isolates (n = 50) were cultured from diverse clinical diagnostic specimens derived from cattle (n = 25), goats (n = 19), and sheep (n = 6). All 50 isolates were recovered from epidemiologically unrelated specimens submitted to the Texas A&M Veterinary Medical Teaching Hospital (VMTH; College Station, TX) between 2010 and 2017.

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Samples were inoculated onto trypticase soy agar supplemented with 5% sheep blood (MilliporeSigma, St. Louis, MO) and incubated for 48 h at 37°C in 5% CO_2. Identification of 35 isolates (25 bovine, 8 caprine, and 2 ovine) collected in 2010–2015 was based on Gram stain, colony morphology, biochemical analyses (RapID CB PLUS system, Remel, Lenexa, KS), the Christie–Atkins–Munch–Peterson (CAMP) test, and PCR detection of the pyolysin gene (*plo*).[7] *T. pyogenes* ATCC 19411 was used as a reference strain. The CAMP test was carried out on the 35 isolates using *Staphylococcus aureus* strain ATCC 25923. Identification of 15 isolates recovered from 11 caprine and 4 ovine specimens in 2015–2017 was performed via matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS; Microflex LT, Bruker Daltonics, Billerica, MA) and the *plo*-specific PCR. The mass spectrometer was calibrated for molecular weights with a range of 3,637–16,952 Da prior to sample testing utilizing the bacterial test standard (Bruker Daltonics) according to the manufacturer's recommendations.

Genomic DNA was extracted (QIAprep spin miniprep kit, Qiagen, Valencia, CA) from 24-h cultures of *T. pyogenes* isolates grown in brain–heart infusion broth (MilliporeSigma) according to the manufacturer's instructions. Bacterial isolates were first screened for the presence of the *Arcanobacterium haemolyticum* phospholipase D (*pld*) gene via conventional PCR, using previously developed primers[5]: forward AhF (5'-ATGTACGACGATGAAGACGCG-3') and reverse AhR (5'-GCTTCCTTGTCGTTGAGATTATTAGC-3'). The PCR program was carried out as follows: denaturation at 95°C for 4 min followed by 30 cycles of 1 min at 95°C, 1 min at 60°C, and 1 min at 72°C; and 1 step of 7 min at 72°C. The *pld*-negative isolates were examined for 8 known and putative virulence genes, utilizing previously developed PCR protocols and primers.[7,17] The PCR program was performed as follows[15]: denaturation at 95°C for 4 min; 35 cycles of 94°C for 1 min, annealing for 30 s at different temperatures (60°C for *nanH*-, *nanP*-, *plo*-, *cbpA*-, and *fimC*-specific PCRs; and 57°C for *fimA*- and *fimG*-specific PCRs),[7,17] 72°C for 3 min; and a final extension at 72°C for 7 min. All amplifications were carried out in 50 µL of the following reaction mixture: 10 pmol of each primer (0.5 µL each), 10 mM of each dNTP (N0447S, New England BioLabs, Ipswich, MA), 1× *Taq* polymerase buffer (B9004S, New England BioLabs), 1 U of *Taq* DNA polymerase (M0273L, New England BioLabs), and ~50 ng of genomic DNA. The PCR products were separated by electrophoresis through a 2% agarose gel in Tris–acetate–EDTA buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA; Thermo Fisher Scientific, Lenexa, KS), stained with ethidium bromide (MilliporeSigma), and visualized using an Alpha Imager HP (Alpha-Innotech, San Leandro, CA).

A fragment of the 16S ribosomal (r)RNA gene was amplified utilizing the universal primers UNF (5'-GAGTTTGATCCTG GCTCAG-3') and UNR (5'-GGACTACCAGGGTATC TAAT-3') targeting bases 9–27 and 805–786 of the 16S rRNA gene of *Escherichia coli*, respectively.[1] PCR was performed in 50 µL of a reaction mixture containing 10 pmol of each primer, 10 mM each dNTP (N0447S, New England BioLabs), 1x *Taq* polymerase buffer (B9004S, New England BioLabs), 1 U of *Taq* DNA polymerase (M0273L, New England BioLabs), and ~50 ng of genomic DNA. The program was composed of an initial denaturation at 95°C for 4 min followed by 35 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 30 s, and a final extension at 72°C for 7 min. The amplicons of expected sizes were further sequenced (Molecular Cloning Laboratories, San Francisco, CA), and the sequence results were verified via BLASTn (http://blast.ncbi.nlm.nih.gov). The sequences of the present and previous study[15] were imported into CLC Genomics Workbench 8.0.2 (https://goo.gl/urWqpy). Multiple sequence alignment (https://goo.gl/oh67Fm) was then performed on the sequences, and the resulting alignments were used to construct a neighbor-joining tree using Kimura substitution model with 2,000 bootstrap replicates. The 2-tailed Fisher exact test was used for comparison of virulence gene distribution between isolates from large and small ruminants. A p value of <0.05 was considered significantly different.

Results

Identification of 35 isolates collected from cattle (n = 25), goats (n = 8), and sheep (n = 2) in 2010–2015 required both biochemical and molecular testing. The use of the RapID CB PLUS system demonstrated that all 35 isolates were positive for glucose and ribose utilization. In contrast, sucrose and maltose were utilized by 26% and 86% of the isolates, respectively. The 35 isolates were positive for α-glucosidase and negative for β-glucosidase activity. Moreover, all of the isolates showed positive results for β-D-glucosaminidase, glycosidase, and β-galactosidase, as well as testing positive for proline, leucyl, and leucine aminopeptidase. One of 35 isolates had a negative result for pyrrolidine aminopeptidase (Supplementary Table 1). All 35 isolates were catalase-negative. Only a single isolate produced yellow pigment. A portion of isolates showed fatty acid esterase (91%), tryptophan aminopeptidase (57%), and phosphatase (54%) activity. Nitrate reductase and urease were present in only 3% of the isolates (Supplementary Table 1). As a result, the RapID CB PLUS system identified 35 isolates as *A. haemolyticum* with the probability of >99%. However, the reverse CAMP test results were negative for the 35 isolates. Furthermore, an amplicon of 528 bp observed for *A. haemolyticum* ATCC BAA-1784 was not detected in any of the isolates. In contrast, the 35 clinical isolates, as well as *T. pyogenes* ATCC 19411, were positive by PCR for the *plo* gene. The *plo*-specific PCR was negative for *A. haemolyticum* strain ATCC BAA-1784. Thus, despite the biochemical test results, the 35 isolates were identified as *T. pyogenes*. The 11 caprine and 4 ovine isolates collected in 2015–2017 were identified as *T. pyogenes* by MALDI-TOF MS and positive *plo*-specific PCR results.

Table 1. Frequency of detection of known and putative virulence genes in 50 isolates of *Trueperella pyogenes.*

Genotype number	Genotype	No. of isolates	Isolates
I	*plo nanH nanP cbpA fimA fimC fimE fimG*	1 (2)	B53-006
II	*plo nanH nanP fimA fimC fimE fimG*	3 (6)	B28-001, C43-039, C62-037
III	*plo nanH nanP fimA fimC fimE*	9 (18)	B32-070, B45-059, B46-008, B57-062, B57-063, C42-002, C45-040, C49-008, C66-086
IV	*plo nanH nanP fimA fimE*	6 (12)	B41-056, B44-047, C49-012, C64-018, C66-081, C67-061
V	*plo nanH nanP fimA fimC*	6 (12)	C55-097, C62-055, O49-050, O57-017, O66-023, O67-048
VI	*plo nanH fimA fimC fimE*	2 (4)	B24-017, C45-041
VII	*plo nanP cbpA fimA fimC fimE*	1 (2)	B45-061
VIII	*plo nanP fimA fimC fimE fimG*	1 (2)	B48-049
IX	*plo nanP fimA fimC fimE*	6 (12)	B24-034, B31-037, B44-039, B44-100, B45-100, B57-070
X	*plo nanP fimA fimE*	1 (2)	C40-018
XI	*plo nanP fimC fimE*	2 (4)	B55-062, B56-098
XII	*plo cbpA fimA fimE*	1 (2)	B45-008
XIII	*plo fimA fimC fimE fimG*	3 (6)	B36-077, B40-004, B51-078
XIV	*plo fimA fimC fimE*	1 (2)	B54-012
XV	*plo nanH nanP fimA fimC fimG*	1 (2)	O60-018
XVI	*plo nanH nanP fimA*	1 (2)	C62-045
XVII	*plo nanH nanP fimC fimE*	2 (4)	C63-010, C63-011
XVIII	*plo nanH nanP fimA fimG*	1 (2)	C63-017
XIX	*plo nanH fimA fimC*	1 (2)	O67-036
XX	*plo nanH nanP fimE*	1 (2)	C67-077

Numbers in parentheses are percentages.

The clinical isolates displayed variable profiles of virulence factors (Table 1); 20 different genotypes were identified. Genotype III (*plo*/nanH/nanP/*fimA*/*fimC*/*fimE*) was the most dominant genotype and was detected in 18% of the isolates. Genotypes IV (*plo*/*nanH*/*nanP*/*fimA*/*fimE*), V (*plo*/*nanH*/*nanP*/*fimA*/*fimC*) and IX (*plo*/*nanP*/*fimA*/*fimC*/*fimE*) were equally represented, containing 12% of respective *T. pyogenes* isolates. Most genotypes, including the genotype that contained all 8 genes (genotype I), were singly represented (genotypes VII, VIII, X, XII, XIV–XVI, XVIII–XX; Table 1).

Phylogenetic analysis of partial sequences of the 16S rRNA gene was performed on 50 sequences of *T. pyogenes* isolates, 46 obtained from our study and 4 from a prior study[15] (Fig. 1). The 4 isolates (808, 836, 868, and 886) from the prior characterization study represented a total of 25 *T. pyogenes* isolates recovered from European bison.[15] The analysis also included sequences of other *Trueperella* and *Arcanobacterium* species as additional references. As a result, the *T. pyogenes* isolates were clustered with the reference strain, *T. pyogenes* ATCC 19411, which further supported the identification of the clinical isolates as *T. pyogenes* (Fig. 1). All 16S rRNA sequences of *T. pyogenes* isolates tested displayed 99–100% identity to each other. Eight sequences of *T. pyogenes* isolates from our study that represented detectable variations were deposited in the GenBank database under the following accessions: KX592201 (O57-017), KX592202 (O49-050), KX592203 (C49-012), KX592204 (C42-002), KX592205 (B57-062), KX592206 (B53-006), KX592207 (B45-059), and KX592208 (B31-037).

Discussion

Initially, the 35 clinical isolates of *T. pyogenes* collected in 2010–2015 were incorrectly identified as *A. haemolyticum* by the RapID CB PLUS system. The isolates did not display a reverse positive CAMP reaction, which is consistently observed for *A. haemolyticum*. Moreover, the synergistic CAMP test results were negative for all 35 isolates and *T. pyogenes* ATCC 19411. The negative CAMP test is a typical feature of *T. pyogenes*,[19] despite the fact that enhancement of staphylococcal hemolysis by *T. pyogenes* is occasionally observed.[6,15,18] The isolates were further examined for the presence of *pld*, a phospholipase D–encoding gene that is specific for *A. haemolyticum*.[5] An amplicon of 528 bp observed for *A. haemolyticum* ATCC BAA-1784 was not detected in any of the isolates. In contrast, the 35 clinical isolates and *T. pyogenes* strain ATCC 19411 tested positive for *plo*, the gene that was previously detected in all

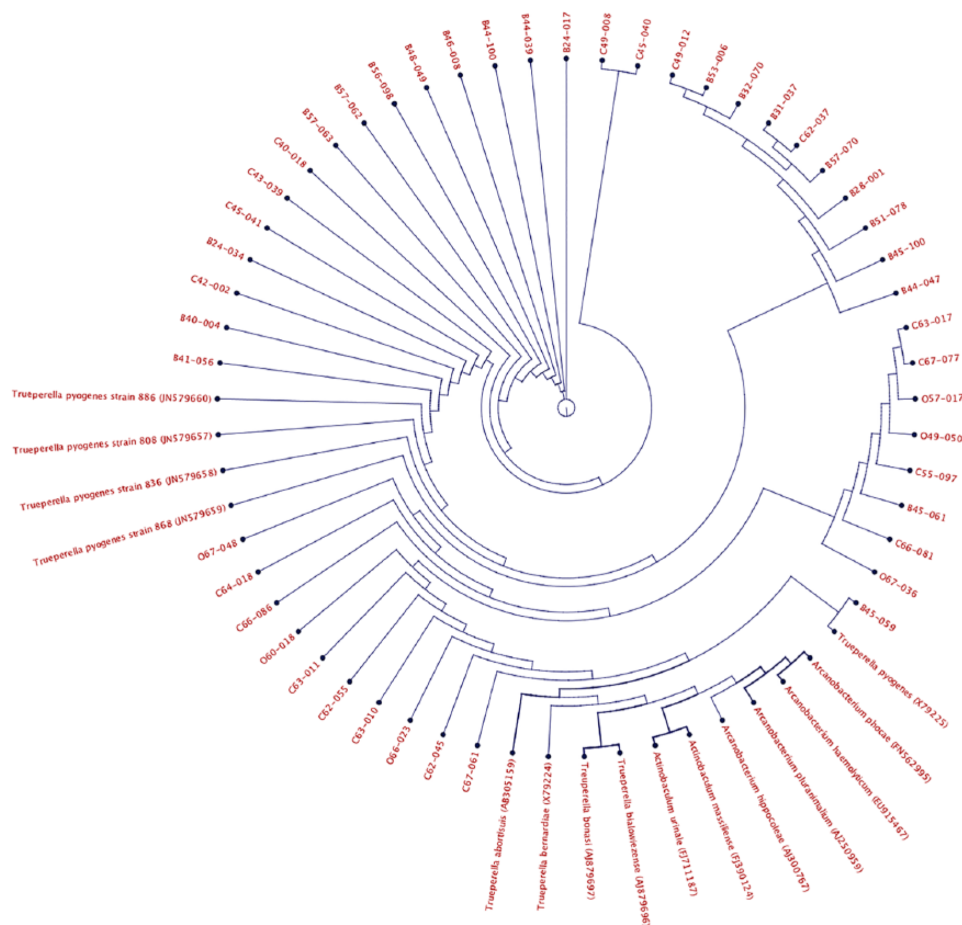

Figure 1. Phylogenetic analysis of 16S ribosomal (r)RNA gene partial sequences of *Trueperella pyogenes* and other related species. Phylogenetic analysis of partial sequences of 16S rRNA gene was performed on 46 *Trueperella pyogenes* isolates in our study. The analysis also included various *Trueperella* and *Arcanobacterium* species as references; as well as 4 *T. pyogenes* isolates, previously isolated from European bison (isolates 808, 836, 868, and 886).[15]

T. pyogenes isolates of various origin.[6,15–17,21] Identification of the 15 isolates recovered in 2015–2017 was performed via MALDI-TOF MS, with confirmation by positive *plo*-specific PCR results. As a result, a total of 50 clinical isolates were identified as *T. pyogenes*.

Adhesion of *T. pyogenes* to host cells is a complex process that involves multiple molecules. CbpA is a collagen-binding protein A that allows *T. pyogenes* to adhere to collagen types I, II, and IV.[4] Despite an important role of CbpA in cell adhesion, the frequency of the *cbpA* gene is highly variable among *T. pyogenes* isolated from various ruminants. Two prior studies demonstrated a high prevalence of the *cbpA* gene among *T. pyogenes* isolates of ruminant origin with ~48% ($n = 45$) and 100% ($n = 57$) of bovine isolates carrying the *cbpA* gene.[4,17] However, in our work, *cbpA* was the least represented gene and was harbored by only 3 bovine isolates (Table 1). The low carriage among ruminant isolates was also noticed previously, being detected in only 1.4% ($n = 72$), 1.5% ($n = 66$), and 7% ($n = 29$) of large ruminant isolates.[3,6,16] Interestingly, the lack of *cbpA* among caprine and ovine isolates in our study is consistent with findings of prior studies in which all tested isolates from goats and sheep tested negative for *cbpA*.[6,12,14]

In addition to CbpA, *T. pyogenes* isolates can express neuraminidases H (NanH) and P (NanP), molecules that are also involved in bacterial adhesion.[8] The occurrence of genes encoding NanH and NanP have been shown to vary 40% and 64%, respectively, to 100% in bovine *T. pyogenes* isolates.[6,8,15,17] Our study demonstrated that *nanH* and *nanP* were present in 68% and 84% of the isolates, respectively (Table 1). Either one or both genes were detected in 38% and 62% of the isolates, respectively. Interestingly, most of the isolates from small ruminants (88%) contained both genes. In contrast, the *T. pyogenes* isolates negative for the 2 genes were only of bovine origin. Two previous studies showed that, out of 21 isolates recovered from small ruminants, only a single isolate of ovine origin did not possess either *nanH* or *nanP* genes.[10,12] The lack of the 2 neuraminidase-encoding

genes was also observed in at least 9 European bison isolates, among which the genotype lacking both genes, *plo*/*fimA*/*fimC*, was found to be predominant.[15] Furthermore, *T. pyogenes* isolates negative for both *nanH* and *nanP* were identified in 5% of the isolates (n = 61) whose origin was not specified.[6] Among *T. pyogenes* isolates from mastitic milk of dairy cows, ~36% of the isolates lacked both genes.[20] Yet, in another study, no neuraminidase-deficient genotypes were identified in any of 65 *T. pyogenes* isolates cultured from cranial abscesses of white-tailed deer[3]; the *plo*/*nanH*/*nanP*/*fimA*/*fimC*/*fimE*/*fimG* genotype that contained both *nanH* and *nanP* genes was detected in 48% of these isolates.[3] Thus, the prevalence of *nanH* and *nanP* varies greatly among clinical isolates of ruminant origin. Overall, identification of *nanH*/*nanP*/*cbpA*-deficient genotypes suggests that molecules other than neuraminidase and CbpA may contribute to adhesion properties of *T. pyogenes*.

Fimbriae are filamentous surface appendages that are involved in cell-to-cell or cell-to-surface adherence of various bacteria. Fimbriae-encoding genes (*fimA, fimE, fimC*, and *fimG*) are consistently detected in *T. pyogenes* isolates.[3,15,17] In our study, *fimA* was among the most frequently detected genes and was found in 90% of the isolates (Table 1). Interestingly, 2 bovine and 3 caprine isolates lacked the *fimA* gene. The *fimA*-negative genotype was also earlier observed in ~9% and 36% of *T. pyogenes* isolates from metritis and non-metritis cows, respectively.[16] The significance of the differences between the 2 groups remains uncertain.[6] In contrast, all other genetically characterized isolates recovered from small and large ruminants including goats (n = 12, total number of isolates tested), sheep (n = 9), European bison (n = 25), dairy cows (n = 57), and deer (n = 66) contained the *fimA* gene.[3,12,14,15,17]

The *fimE* gene was harbored by 80% of the isolates. The high prevalence of the *fimE* gene (98%) was also noted in other studies.[3,17] The carriage frequency of *fimC* is generally lower than that of *fimA* and *fimE* and may range from 67% to 88% (78% in our study).[3,15,17] The *fimG* gene was least represented among the fimbrial genes tested and was present in only 20% of our isolates. The low occurrence of *fimG* is supported by a prior study, in which the gene was detected in 24% of *T. pyogenes* isolated from European bison.[15] However, it was also shown that 67% and 68% of *Trueperella* isolates recovered from dairy cows and white-tailed deer, respectively, carried the *fimG* gene.[3,17]

In our study, the fimbriae-encoding gene, *fimE*, was detected more frequently in bovine isolates (p = 0.0006) than in caprine and ovine isolates. In contrast, the *nanH* gene was detected with higher frequency in caprine and ovine isolates (n = 24) than bovine isolates (n = 10; p = 0.0001). However, the data from a 2017 study that analyzed 57 and 7 isolates from large (cattle, n = 57) and small (n = 7) ruminants, respectively, does not support the identified correlation for either *fimE* or *nanH* (p = 1.0000).[12] In our study, genotype V (*plo*/*nanH*/*nanP*/*fimA*/*fimC*) was only detected in caprine and ovine isolates (n = 6); whereas genotype IX (*plo*/*nanP*/*fimA*/*fimC*/*fimE*) was solely present in the isolates of bovine origin (n = 6; p = 0.0223). Further studies involving a greater number of *T. pyogenes* isolates from small and large ruminants are warranted to confirm the identified association of the 2 genotypes to the respective animal species.

Studies involving virulence factors are important for investigation of molecular epidemiology and pathogenicity of various pathogens.[12] Our study contributes to the understanding of phenotypic and genotypic diversity that exist among *T. pyogenes* isolates recovered from large and small ruminants and, overall, reinforces the importance of characterization of more clinical isolates in order to better understand the pathogenesis of this bacterium in different animal species.

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Declaration of conflicting interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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