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Detection of the Novel *optrA* Gene Among Linezolid-Resistant Enterococci in Barcelona, Spain

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The purpose of this study was to describe the presence of the novel *optrA* gene among clinical isolates of enterococci in a Spanish teaching hospital (May 2016–April 2017). *optrA* and *cfr* genes were screened by PCR in all isolates showing linezolid minimal inhibitory concentration (MIC) ≥4 mg/L. The genetic relatedness of the isolates, the presence of resistance and virulence genes, and the genetic environment of *optrA* were assessed by whole-genome sequencing (WGS). Six of 1,640 enterococci had linezolid MIC ≥4 mg/L. Among them, the *optrA* gene was detected in five *Enterococcus faecalis* isolated from unrelated patients. Although none of them had received linezolid or chloramphenicol, all had antecedents of recent quinolone consumption. WGS analysis revealed the existence of two different genotypes: ST585 and ST474. *cfr* was not detected in any of the isolates. No mutations were detected among the 23S ribosomal RNA and the ribosomal proteins L3, L4, and L22. Both genotypes also carried genes related to aminoglycoside, lincosamide, macrolide, phenicol, and tetracycline resistance. Detection of *optrA* in a setting with low linezolid consumption and among patients without antecedents of oxazolidinone therapy is of concern.

Keywords: linezolid, *optrA*, *cfr*, *enterococci*, chloramphenicol, *Enterococcus faecalis*

Introduction

Linezolid resistance is emerging as a problem in the treatment of multidrug-resistant Gram-positive organisms, particularly methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecium*.[1,2] Among enterococci, linezolid resistance is mainly mediated by chromosomal mutations in domain V of the 23S ribosomal RNA (rRNA).[3] However, a transferable oxazolidinone resistance gene (*cfr*), which encodes a methyltransferase that modifies the 23S rRNA, was described in 2001.[4] This gene confers resistance to linezolid, phenicols, lincosamides, pleuromutilins, and streptogramin A. Recently, a novel mechanism (*optrA*) was described in *Enterococcus* spp.,[5–12] *Staphylococcus* sciuri,[13,14] and *Streptococcus suis*,[15] indicating its ability to spread to different genus. *optrA* encodes an ABC-F protein that protects the bacterial ribosome from the antibiotic inhibition, has the ability to spread through plasmids, and confers resistance to linezolid and phenicols.[5,16]

In our setting, Hospital Universitari de Bellvitge (HUB), linezolid resistance is rare (<1% in both enterococci and staphylococci) and the *cfr* gene has been sporadically detected in *S. aureus*.[17] The purpose of this study was to screen for the presence of transferable linezolid resistance among linezolid-resistant clinical isolates of enterococci in a tertiary care hospital in Barcelona, Spain.

Materials and Methods

Study setting, bacterial isolates, and antimicrobial susceptibility testing

This study was performed at HUB, a 700-bed teaching hospital located in the urban area of Barcelona, Spain. From May 2016 to April 2017, all enterococci isolates obtained from clinical samples were routinely identified by Matrix-Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) (Bruker Daltonik GmbH, Bremen, Germany) and tested for antimicrobial susceptibility by microdilution (Microscan®; Beckman Coulter, Brea, CA) following the Clinical and Laboratory Standards Institute (CLSI) recommendations and criteria.[18] The linezolid minimal inhibitory concentration (MIC) breakpoints were as follows:

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susceptible ≤2 mg/L, intermediate 4 mg/L, and resistant ≥8 mg/L. Isolates showing linezolid MIC ≥4 mg/L were further studied by microdilution, using Sensititre Rapmyco® plates (Thermo Fisher Scientific, Waltham, MA) with linezolid concentrations ranging from 1 to 32 mg/L.

Molecular characterization

Linezolid-resistant isolates were screened for both *cfr* and *optrA* genes by PCR, as described elsewhere.[7] Whole-genome sequencing (WGS) was performed in those *optrA*-positive isolates. Libraries were prepared (NexteraXT®) and multiplexed in an MiSeq run (150 bp paired-reads). Reads were processed using Geneious 9.1.7 (Biomatters, Auckland, New Zealand): duplicate reads were removed, ends were trimmed, and reads were *de novo* assembled using the Geneious assembler with default settings (medium sensitivity). The genetic environment of *optrA* was *in silico* studied comparing those contigs containing the *optrA* gene with those previously published. The existence of resistance and virulence genes and the genetic relatedness of the isolates were studied through the online tools Resfinder 2.1, Virulence finder 1.5, and CSI phylogeny (www.genomicepidemiology.org/, last accessed June 15). This last analysis included, for comparative purposes, all previously sequenced *optrA*-positive *Enterococcus faecalis* strains (Supplementary Table S1; Supplementary Data available online at www.libertpub.com/mdr). Data from WGS were also used to search for chromosomal mutations related to oxazolidinone (23S rRNA and ribosomal proteins L3, L4, and L22) and quinolone (GyrA and ParC proteins) resistance. *E. faecalis* ATCC 29212 (accession number CP008816) was used as reference strain. Raw data were deposited to the European Nucleotide Archive (www.ebi.ac.uk/ena) with accession numbers ERS1788479–1788483.

Clinical data

Clinical data of patients were retrospectively reviewed from electronic records. Data included age, sex, admission ward, previous antimicrobial therapy, treatment, and outcome. This study was approved by the Clinical Research Ethics Committee of HUB (PR354/17). As no intervention was involved and patients' identification was anonymized, written informed consent was considered not necessary.

Results

During the study period, 1,640 enterococci isolates were collected from clinical samples of 1,249 patients. Table 1 gives the species and the linezolid MICs of the studied population. Overall, 1,634 isolates (99.6%) had MIC ≤2 mg/L and only 6 *E. faecalis* (0.4%) were linezolid nonsusceptible (MIC ≥4 mg/L). In our series, *E. faecalis* isolates had significantly higher linezolid MICs than *E. faecium* ($p < 0.01$). After PCR screening, the *optrA* gene was detected in five of these nonsusceptible isolates, all showing chloramphenicol MIC of >256 mg/L (Table 2). We did not detect the *cfr* gene in any of the isolates. All five isolates were recovered from urine samples of five male patients admitted to three different wards. Two patients had clinical symptoms of urinary tract infection and were successfully treated with aminopenicillins (ampicillin and amoxicillin). The remaining three patients were asymptomatic and their isolates were obtained from low urinary bacterial counts. Consequently, these isolates were considered colonizing and the patients did not receive antimicrobial therapy. As we did not find any epidemiological relationships between these five patients, these acquisitions were considered unrelated. Although none of them had received oxazolidinones or phenicols, all had antecedents of recent (<6 months) antimicrobial therapy.

Table 1. *Enterococcus* Species and Linezolid MICs of the Study Population

Enterococcus *species*	*Linezolid MIC (mg/L), number of isolates (%)*			
	<1	*2*	*4*	*>4*
E. avium	12 (85.7)	2 (14.3)		
E. casseliflavus	2 (66.7)	1 (33.3)		
Enterococcus faecalis	703 (59.0)	483 (40.5)	2 (0.2)	4 (0.3)
Enterococcus Faecium	289 (70.0)	124 (30.0)		
E. gallinarum	4 (66.7)	2 (33.3)		
E. hirae	1 (25.0)	3 (75.0)		
E. raffinosus	4 (100)			
Other	2 (50.0)	2 (50.0)		
All	1,017 (62.0)	617 (37.6)	2 (0.1)	4 (0.2)

Only species accounting for more than two isolates are given.
CLSI linezolid MIC breakpoints were as follows: susceptible ≤2 mg/L, intermediate 4 mg/L, and resistant ≥8 mg/L.[18]
MIC, minimal inhibitory concentration; CLSI, Clinical and Laboratory Standards Institute.

As deduced from WGS, isolates belonged to two sequence types (ST474 and ST585) that were also discriminated by the single-nucleotide polymorphism (SNP) analysis: SNP differences were 502 among ST474 isolates, ranged from 140 to 300 among ST585 isolates, and were ~15,000 between both groups (Supplementary Table S2). Overall, the WGS analysis of *E. faecalis* carrying *optrA* displayed high genetic diversity, including, as defined by multilocus sequence typing (MLST), 12 different genotypes (Fig. 1 and Supplementary Table S1). With regard to the isolates presented in this study, although ST585 strains carrying *optrA* have been described from human samples in China,[5,6] we could not find any report of ST474. This sequence type that was added to the MLST webpage (www.mlst.net) from the Chinese Guangdong province (liver sample from a pig) shares four of seven MLST alleles with ST59. Interestingly, although this ST59 was first reported from a Spanish pig in 2001,[19] it has been recently detected among *optrA*-positive isolates in China (human and animal sources)[5,6] and Colombia (poultry meat).[8] Data from WGS confirmed this relationship showing that ST474 isolates were related to the ST59 Colombian isolates (12E and 34E) and to an additional isolate collected in Malaysia in 2012 (Enfs85).

Investigation of acquired resistance genes revealed that all isolates carried genes encoding resistance to tetracyclines, macrolides, lincosamides, streptogramin B, and phenicols (Table 2). Four isolates shared the OptrA wild type, originally described in *E. faecalis* E394, whereas one ST474 isolate had an amino acid substitution (Tyr176Asp).[6] All strains harbored *cat* (*cat A-8*) and *fexA* genes, both related

Table 2. Clinical and Microbiological Characteristics of *optrA*-Positive *Enterococcus faecalis* Isolates

ID	*Isolation date/ward*	*Age (years)*	*Previous antibiotic therapy*	*ST*	*Acquired resistance genes*	*OptrA variant*	*Virulence factors*[a]	*LZD*	*MIC (mg/L)*						
									CHL[b]	*AMP*	*CIP*	*TEC*	*VAN*	*HLGR*	*HLSR*
1	May 29/pneumology	76	NOR	585	*lsa(A), erm(B), cat, fexA, optrA, tet(M), tet(L),str*	*Wild type*	*cylA, cylB, cylL, cylM,*	8	>256	<1	>2	<1	<1	<500	>1,000
2	Oct 20/urology	68	CIP CXM	585	*lsa(A), erm(B), cat, fexA, optrA, tet(M), tet(L)* ***aadE, aac(6′)-aph(2″), aph(3′)-III, ant(6)-Ia, lsa(A), lnu(B), dfrG,*** str	*Wild type*	*cylA, cylB, cylL, cylM, espfs*	8	>256	<1	>2	<1	2	>500	>1,000
3	Nov 21/nephrology	69	CIP SXT	585	*lsa(A), erm(B), cat, fexA, optrA, tet(M), tet(L),str*	*Wild type*	*cylA, cylB, cylL, cylM*	8	>256	<1	>2	<1	<1	<500	>1,000
4	Nov 21/urology	68	CIP CLI	474	*lsa(A), lnu(B),* ***erm(A)****, erm(B),* ***spc****, cat, fexA, optrA, dfrG, tet(M), tet(L),* ***aadE, aph(3′)-III, ant(6)-Ia****,* ***aac(6′)-aph(2″),****str*	Tyr176Asp	*hylA, hylB*	8	>256	<1	>2	<1	<1	>500	>1,000
5	Dec 5/nephrology	62	CIP	474	*lsa(A), lnu(B),* ***erm(A)****, erm(B),* ***spc****, cat, fexA, optrA,* ***dfrG****, tet(M), tet(L),* ***aac(6′)-aph(2″), aadE, aph(3′)-III, ant(6)-Ia***	*Wild type*	*hylA, hylB*	8	>256	<1	>2	<1	<1	>500	>1,000

Differences in resistance genes are highlighted in bold.

[a]Only differences are given. All isolates harbored *ace, agg, cad, camE, cCF10, cOB1, ebpA, ebpB, ebpC, efaAfs, elrA gelE, srtA, tpx* and *fsrB* genes.

[b]*E*-test.

AMP, ampicillin; CHL, chloramphenicol; CIP, ciprofloxacin; CLI, clindamycin; CXM, cefuroxime; HLGR, high-level gentamicin resistance; HLSR, high-level streptomycin resistance; LZD, linezolid; MIC, minimal inhibitory concentration; NOR, norfloxacin; ST, sequence type; SXT, co-trimoxazole; TEC, teicoplanin; VAN, vancomycin.

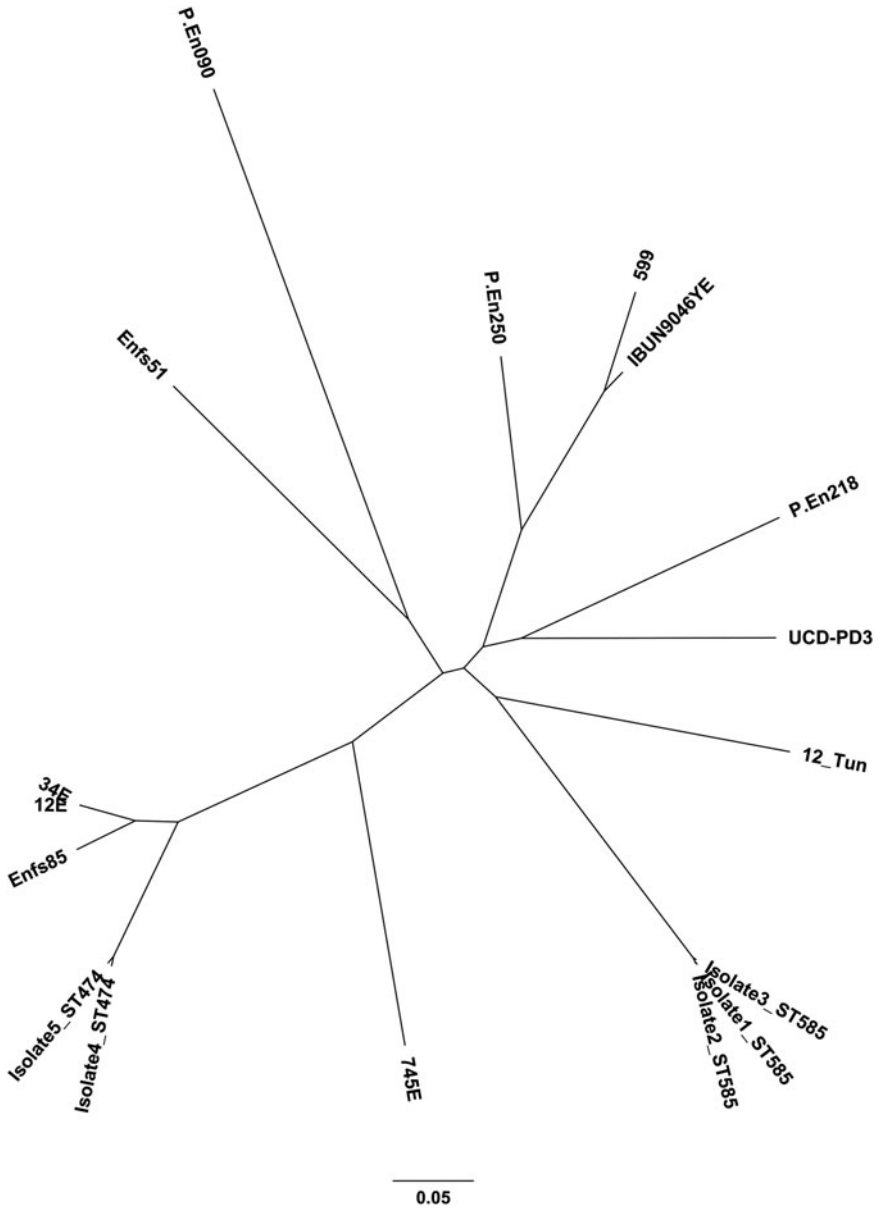

FIG. 1. SNP phylogenetic tree of *Enterococcus faecalis* carrying *optrA*. The scale bar represents the genetic divergence between isolates (number of SNPs/total number of polymorphic sites). The figure includes isolates reported in this study (isolates 1–5), previously published isolates, and WGS sequences of *optrA*-positive *E. faecalis* obtained after performing a blast search in the NCBI public database (Table S1). SNP, single-nucleotide polymorphism; WGS, whole-genome sequencing.

to phenicol resistance. No mutations were detected among the 23S rRNA and ribosomal proteins L3, L4, and L22. One ST585 isolate and all ST474 isolates carried the *aac(6′)-aph(2″)* gene, conferring high-level resistance to gentamicin. All isolates showed amino acid substitutions related to quinolone resistance[20] in ParC (Ser80Ile) and GyrA (Ser83Phe and Glu87Gly in ST585 and ST474 isolates, respectively). Both genotypes shared a high number of virulence factors including genes related to adhesion (*ace, agg, efaAfs,* and *elrA*), biofilm production (*ebpA, ebpB,* and *ebpC*), proteases (*gelE* and *srtA*), protection against oxidative stress (*tpx*), and genes involved in quorum sensing (*cad, camE, cCF10, cOB1,* and *fsrB*). Main differences were the existence of the cytolysin operon (*cylA, cylB, cylL*, and *cylM* genes) only in ST585 and the presence of hyaluronidase genes *hylA* and *hylB* only in ST474. As both STs were responsible for episodes of not only infection but also colonization, we could not hypothesize about the importance of these factors in the pathogenesis of isolates.

Finally, the genetic environment of the *optrA* gene was *in silico* compared with previously published data and, in agreement with that, all ST585 isolates harbored a genetic context identical to that reported from plasmid pXY17 (described in ST585 isolates from China). Isolate 4 (ST474) showed a structure related to plasmid p10-2-2, which is in agreement with the fact that this plasmid was found in an ST59 isolate, genetically related to ST474.[10] Isolate 5 showed truncated contigs and the region could only be partially revealed (Fig. 2).

Discussion

The frequent detection of *optrA* among Chinese isolates of animal origin suggests their selection by the use of antibiotics in animals (florfenicol), or indirectly by other antibiotics for human uses whose resistance mechanisms are harbored by the same plasmids. A previous study found a high prevalence of linezolid resistance genes in soils next to

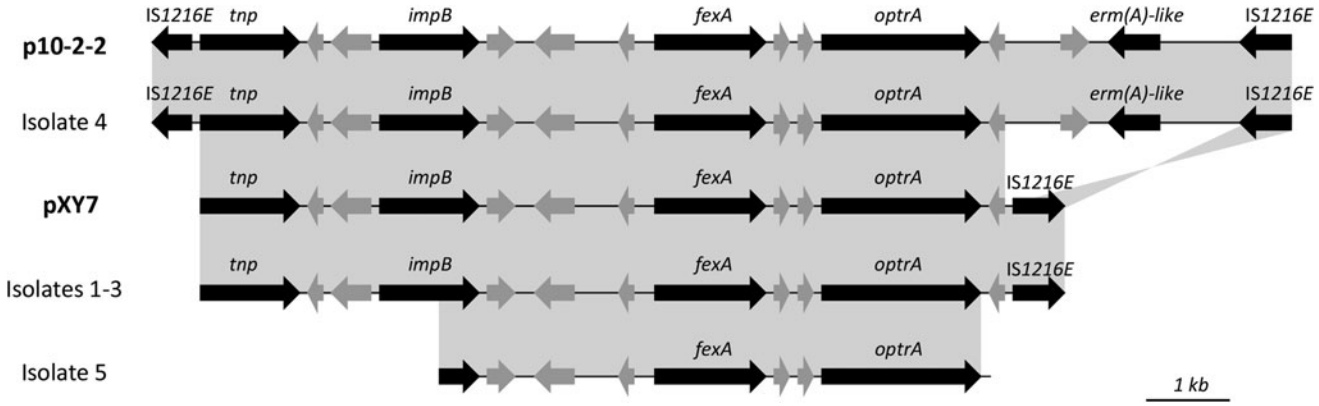

FIG. 2. Graphic representation of the genetic environment of optrA containing regions. Contigs containing *optrA* were compared with previously published regions and then reads were mapped against these regions. Isolates 1, 2, and 3 (ST585) shared the same genetic environment described in plasmid pXY17 and isolate 4 (ST474) showed a structure related to plasmid p10-2-2. The structure of the region containing *optrA* of isolate 5 could only be partially revealed.

pig farms in China, including both *cfr* and *optrA* genes, establishing a clear link between resistance and livestock farming.[21] In addition, the recent report of *optrA* from a human-related lineage of *E. faecalis* (ST86) in urban wastewater (Tunisia) suggests the existence of effective dissemination in the human setting.[22] Data from European countries and the United States are still scarce. In Europe, a few *optrA*-positive enterococci isolates have been reported.[7,11,12,23] A recent multicenter study carried out on isolates from 42 countries, excluding the United States, detected the *optrA* gene in 9 isolates (8 *E. faecalis* and 1 *S. gallolyticus*) from a large sample of 854 enterococci and 469 viridans-group streptococci screened.[24] In a linezolid surveillance program in the United States, the *optrA* gene was found in 2 *E. faecalis* of 973 enterococci screened.[25]

This is the first detection of *optrA* in Spain, as far as is known. As linezolid consumption is low in our setting, and none of the patients harboring *optrA*-positive isolates received linezolid or chloramphenicol therapy, our findings are of concern. As reported, the coexistence of other resistance markers in the same mobile genetic elements could justify the role of antimicrobials other than oxazolidinones or phenicols in spreading these strains.[5] Although we were not able to find *optrA* and other resistance genes colocated in the same genetic environment (with the exception of *fexA*), their association cannot be excluded from our data. Therefore, other antimicrobials for human use (*i.e.*, macrolides, quinolones, tetracyclines, or streptomycin) could contribute to the selection of these isolates in the clinical setting. In this respect, it is remarkable that all isolates were resistant to ciprofloxacin and this was because of a nontransferable mechanism. Data on antibiotic consumption from our hospital indicate that, after penicillins, quinolones are the second group of most prescribed antimicrobials (10.74 prescribed daily doses per 100 patient-days in 2015), substantially exceeding the consumption of linezolid (0.48 daily doses per patient-day in 2015). The fact that all isolates were recovered from urine samples and all patients had antecedents of quinolones consumption, which are frequently used in urine tract infections, could indicate that, in our clinical setting, these antibiotics may have played an important role in the selection of these isolates. In fact, quinolones have already been involved in mechanisms of antimicrobial resistance co-selection among Gram-negative bacteria.[26] Although *optrA* has not been clearly linked to any case of clinical failure after linezolid treatment, its presence in the hospital setting is worrisome. As no evidence of chromosomal mutations related to linezolid resistance were detected (that are usually associated to linezolid utilization),[27] it seems that the emergence of *optrA* isolates in our setting is independent of oxazolidinone consumption. In addition, the detection of different genotypes isolated from apparently unrelated patients could be an indirect sign of its dissemination in our region and beyond the hospital setting, which needs to be clarified in further studies.

Our study adds epidemiological information on transferable linezolid resistance. Data obtained from WGS are particularly useful for assessing genetic relatedness among bacteria, allowing data sharing worldwide. The study of previously published WGS sequences of *optrA*-positive *E. faecalis* reveals the existence of multiple lineages and worldwide distribution. Specifically, WGS allowed us to put in context our clinical isolates and to establish genetic relationship between the two of them (isolates 4 and 5), an isolate from animal origin (Malaysia, 2012) and two isolates from poultry meat (Colombia, 2011).[8] WGS is not able to solve the origin of this association. However, the fact that these isolates were obtained from both human and animal origin in several different countries indicates the existence of a wide distribution of *optrA*. As isolates belonging to ST59 have been described before the *optrA* description in animals[19,28] and also in the clinical setting,[29] the ability of this lineage to cause invasive human disease is evident. Actually, this goes in line with the high number of virulence factors detected, most of them also shared by ST585 isolates.

Our study has limitations. As we only included isolates showing linezolid MIC ≥4 mg/L, and *optrA* has been detected in isolates with lower MICs,[5,7,9,13] our findings could underestimate its presence. This could be especially important in environments with higher linezolid consumption and higher resistance rates that could masquerade the presence of this mechanism. In addition, although WGS is a powerful epidemiological tool, complete plasmid sequences are difficult to assemble through short reads giving only partial results that makes the analysis of these genetic elements difficult.[30]

In conclusion, we report the presence of *optr*A among clinical linezolid-resistant isolates in an urban area of Barcelona that is not related to linezolid consumption. Active surveillance and continued efforts to control the use of antimicrobials in veterinary and clinical practice are needed worldwide. Otherwise, the arsenal of available antimicrobial drugs against infections caused by multidrug resistance could be significantly reduced.

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Disclosure Statement

No competing financial interests exist.

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