Original Article

PCR - RFLP patterns for the differentiation of the Fusarium species in virtue of ITS rDNA

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Abstract

Background and Purpose: The *Fusarium* species are among the most important fungi in the medical, veterinary and agricultural fields.

Materials and Methods: In the present study, 172 strains of these fungi have been analyzed. The high molecular weight DNAs were extracted from 23 reference strains as well as from 149 isolated *Fusarium* species. Using the designed nucleotide primers from rDNA of *Fusarium* species, PCR analysis was performed for the amplification of ITS regions. Afterwards, the location of the effective endonuclease enzymes has been evaluated within approximately 930 bp of rDNA sequence.

Results: Through the selected enzymes including; *Hha*I, *Msp*I, *Taq*I and *Faq*I, the mentioned *Fusarium* species have been divided into 33 groups. The first three enzymes were able to classify *Fusarium* species into 23 groups of which 19 groups included one member, one group included two members and three groups included three members of the *Fusarium* species. This study also revealed the possibility in the identification of *F. semitectum*, *F. solani* complex, *F. pseudograminearum*, *F. nisikadoi*, *F. coeruleum* and *F. acuminatum* species by one unique enzyme. In addition, our study indicated the ability of the differentiation of *F. Compactum* from *F. equiseti*.

Conclusion: As Compared to previous studies with more endonuclease enzymes and with limited in identifications, the ITS-RFLP patterns reported here an attempted to evaluate most of the *Fusarium* species successfully.

Keywords: *Fusarium* spp., PCR-RFLP, ITS rDNA

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Introduction

The *Fusarium* species can incite directly the diseases in plants, humans and domesticated animals. The mortality rate for human patients with the systemic *Fusarium* infections is 70% [1]. In addition, *Fusarium* spp. produce secondary metabolites associated with plant diseases, as well as with cancer and other growth defects in humans and domestic animals [2-4].

Fusarium is one of the most heterogenous fungal genera in which the classification of species within this genus is very complicated. Currently, the discrimination of *Fusarium* spp. has been done based on the morphological characteristics such as the shape and the size of the macroconidia, the presence/absence of microconidia and chlamydospores and also the colony morphology. These procedures are time-consuming. They need much effort and an expert staff. Therefore, a rapid and reliable assay for the identification of *Fusarium* spp. would be beneficial. The polymerase chain reaction (PCR) technique is a sensitive, rapid and a reliable diagnosis method in species identification which will enable us to overcome the poor sporulation of the *Fusarium* spp. and its identification [5, 6].

Interestingly, the molecular approaches have been developed for *Fusarium* systematic studies including; Random Amplified Polymorphic DNA (RAPD) analysis, a specific diagnostic PCR primers and DNA sequencing [7-14]. However, the most current methods are

often based on the ribosomal RNA (rRNA) sequences analysis which holds both conserved and variable regions, allowing discrimination at different taxonomic levels [15, 16].

The restriction analysis of PCR-amplified rDNA (rRNA gene) sequences has been shown to be a suitable method for the taxonomic studies in *Fusarium* spp. [17-19]. A few limited results of PCR-RFLP based on the identification of *Fusarium* species have been reported so far in *Martiella*, *Elegans*, *Liseola* and *Sporotrichiella* sections [18, 20-22]. These studies showed that the nucleotide sequences of the ITS regions are useful for identifying *Fusarium* species. The aim of the current study was to evaluate the utility of PCR-RFLP of the ITS region for discriminating the *Fusarium* species.

Material and Methods

Fungal isolates

One hundred seventy-two fungal strains including 23 reference *Fusarium* strains and 149 *Fusarium* isolates obtained from Iranian cereal grains were included in the present study (Table 1) [23]. All the isolates were identified morphologically according to Nelson et al., subcultured on potato dextrose agar (PDA) medium and incubated at 25°C for 1-2 weeks before storage [24].

Table 1. The *Fusarium* reference strains and Iranian isolates used in this study

Fusarium species	Reference strains	Iranian isolates	Total
F. acuminatum	MCR 3231	-	1
F. avenaceum	MCR 8381	1	2
F. babinda	-	1	1
F. camptoceras	-	2	2
F. chlamydosporum	-	1	1
F. compactum	MCR 2800	4	5
F. culmorum	-	2	2
F. dlamini	-	1	1
F. graminearum	MCR 4712, 4927, 6010	-	3
F. heterosporum	-	1	1
F. cf.langsethiae	-	1	1
F .nygamai	MCR 8547	7	8
F. oxysporum	-	6	6
F. poae	MCR 8485, 8486	-	2
F. proliferatum	MCR 8549, 8550	52	54
F. pseudograminearum	MCR 8443	-	1
F. pseudonygamai	-	2	2
F. sporotrichioides	VTT D72014, BBA 10329, MCR 4333, 0043	-	4
F. subglutinans	MCR 8553, 8554	20	22
F. thapsinum	MCR 8557, 6251	-	2
F. tricinctum	-	4	4
F.verticellioides	MCR 8559, 8560, 0826	43	46
F. xylarioides	-	1	1
Total	23	149	172

DNA isolation

Fungal DNA was extracted according to the standard protocols [25, 26]. Except for a few modifications; the harvested mycelial mass was flash-frozen in liquid nitrogen and proceeded to make a fine powder in a porcelain mortar. The powder was suspended in the DNA extraction buffer including 50mM Tris-HCl (pH 8.0), EDTA (50mM), 3% SDS and 50µl of proteinase-K(20mg/ml). Then the suspension was incubated (65°C for 1h) and the cellular debris removed by centrifugation (2500 g for 5 min).

After the addition of 25 µl RNase H (10mg/ml), the suspension was incubated at 37°C for 30 min and extracted once with phenol-chloroform-isoamyl alcohol (25:24:1) and once with chloroform isoamyl alcohol (24:1). After that DNA was precipitated using the equal volume of isopropanol and centrifugation (15000 g for 30 min). Finally, the DNA pellet was washed with 70% ethanol and re-suspended in distilled water after being air dried.

Designing Primer

Forward primer ITS5 (5'GGAAG TAAAAGT CGTAACAAGG3') reported by white et al. [27], and newly designed reverse primer as7 (5'CTTCCCTTTCAACAATTTC AC3') from 28S rDNA region was used for the amplification of *Fusarium* species. Multiple Sequence alignment analysis was performed via MEGA5.1 software.

PCR amplification

Amplification was performed including 2.5 µl of 10X PCR buffer, 0.2 mM of each dNTPs, 0.1 µM of each forward and the reverse primers, template DNA (25ng), and 2.5 U of *Taq* DNA polymerase. The PCR condition was set up in

initial denaturation at 94°C for 2 min, 35 cycles (each of 30s at 94°C, 30s at 59°C, 1 min at 72°C), and a final extension at 72 °C for 7 min. Amplified products were visualized by 1% (w/v) agarose gel electrophoresis in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) stained with ethidium bromide (0.5 ug/ml).

ITS-RFLP analysis

The ITS5-As7 sequences of the various *Fusarium* species obtained from DDBJ/ EMBL/GenBank databases were aligned by MEGA3.1 software. The restriction patterns of the PCR products of the species mentioned above were predicted for each of the known restriction enzymes using the Webcutter online software. The predicted restriction fragments were compared for choosing the best discrimination. Finally, the enzymes *Msp*I, *Hha*I, *Taq*I and *Faq*I were selected (Table 2). Digestion reaction was performed by incubating a 5 µl of PCR product with 2.5 U of enzymes (Fermentas) in a final reaction volume of 15 µl at 37°C for 2h. The restriction fragments were separated by 1.8% agarose gel electrophoresis in TAE buffer for 50 min at 90 V and stained by the ethidium bromide.

Result

Fungal Isolates

A total of 172 *Fusarium* isolates including 23 reference strains and 149 Iranian isolates were analyzed. The isolates that belonged to 23 Fusarium species were as follows: *F. proliferatum (54), F. verticellioides (46), F. subglutinans (22), F. nygamai (8), F. oxysporum (6), F. compactum (5), F. sporotrichioides(4), F. tricinctum (4), F. graminearum (3), F. poae (2), F. camptoceras (2), F. culmorum(2), F. pseudonygamai (2), F. avenaceum (2), F. thapsinum (2), F. acuminatum (1), F. babinda (1), F. chlamydosporum (1), F. dlamini (1), F.heterosporum (1), F. cf.langsethiae (1), F. pseudograminearum (1) and F. xylarioides* (1) (Table 1).

Molecular characterization of the Fusarium Isolates

The size of amplified PCR products was estimated to be 930 bp while using ITS5 and

Table 2. Size of rDNA ITS gene PCR products from a number of *Fusarium* species in accordance to the GenBank / EMBL data library

Species	Size of PCR product (bp)	EMBL accession no.
F. chlamydosporum	895	EU715615
F. sambucinum	913	AY188921
F. poae	915	AY188915
F. venenatum	920	AY188922
F. pseudograminearum	924	DQ459871
F. sporotrichioides	925	AY188917
F. proliferatum	925	GQ167230
F. oxysporum	926	DQ535184
F. semitectum	926	AY633745
F. subglutinans	926	GQ167235
F. culmorum	927	DQ459870
F. crookwellense	927	DQ459869
F. austeroamericanum	927	DQ459837
F. boothii	927	DQ459848
F. brasilicum	927	DQ459860
F. cortaderiae	927	DQ459859
F. lunulosporum	927	DQ459868
F. meridionale	927	DQ459842
F. mesoamericanum	927	DQ459844
F. graminearum	927	DQ459830
F. verticellioides	927	GQ168840
F. asiaticum	928	DQ459835
F. equiseti	928	EU595566
F.armeniacum	930	GQ505462
F. lateritium	939	AY188920
F. tricinctum	943	AY188923

As 7 primer pair. The actual size of PCR products of rDNA region in some species of *Fusarium* is shown in Table 3 in comparison with the existing data in the NCBI. The lowest and the highest number of the fragments were belonged to the digestion of *F. chlamydosporum* and *F. tricinctum* PCR products, respectively. The patterns and the estimated sizes of the restriction fragments that generated four restriction enzymes (*HhaI*, *MspI*, *TaqI* and *FaqI*) (Table 3) revealed that *HhaI* and *FaqI* enzymes showed 7 patterns (A to G), *TaqI* enzyme, 6 patterns (A to F) and the highest number of patterns (14 different patterns) that were obtained via *MspI* enzyme. The selected enzymes divided the *Fusarium* species in 33 groups (Table 3, 4). In the present study, by using three restrictive enzymes including *HhaI*, *MspI*, *TaqI*, the 23 different groups were identified (Table 4). 19 out of them

Table3. Band Patterns (A to N) and their estimated restriction fragment sizes (base pairs) obtained from rDNA ITS digestions

Band Pattern	Enzyme Hha I (Cfo I)	Msp I	Taq I	FaqI
A	530, 315	825, 100	490, 240	700, 220
B	530, 200, 120	710, 120	430, 235	580, 220, 135
C	450, 315	570, 330	340, 235	430, 300, 220
D	450,200,120	525, 330	290, 235	410, 240, 220
E	370, 315, 175	500, 445	290, 235, 145	320, 250, 220, 135
F	370, 200, 170, 120	500, 380	235, 210, 125	300,275, 220, 135
G	>320[1], 250	500, 335, 140	-	>385[1], 185
H	-	445, 375, 100	-	-
I	-	445, 120	-	-
J	-	430, 375	-	-
K	-	430, 300, 120	-	-
L	-	430, 250, 120	-	-
M	-	380, 200	-	-
N	-	300, 160, 120	-	-

1. Considering the short registered sequences recorded in GenBank / EMBL database, cannot completely cut location and length of components identified.

Table 4. Band Patterns (A to N) revealed by restriction analysis of PCR-amplified ITS rDNA region among *Fusarium* isolates

Isolate	Enzyme Hha I	Msp I	Taq I	FaqI	Type
F. dimerum	A	E	B	D	AEBD**
F. nivale(Microdochium nivale)	A	G	B	B	AGBB
F. semitectum(F. incarnatum)	B	A	F	E	BAFE
*F. solani complex**	E	H	A	C	EHAC
F. pseudograminearum[1]	D	A	C	B	DACB
F. austroamericanum[1]	B	A	D	B	BADB
F. sacchari[2]	A	I	C	E	AICE
F. nisikadoi	C	A	C	B	CACB
F. brevicatenulatum	A	L	C	E	ALCE
F. acutatum, F.concentricum[2] *,F. redolens*[3]	E	N	B	B	ENBB
F. venenatum	A	A	D	A	AADA
F. sublunatum	B	L	B	F	BLBF
F. mangifera	A	L	B	B	ALBB
F. concolor	F	C	C	B	FCCB
F. coeruleum	G	E	G	G	GEGG
F. beomiform	F	N	B	B	FNBB
F. ambrosium	E	H	D	C	EHDC
F. tricinctum, F. avenaceum, F. heterosporum	F	C	B	B	FCBB
F. verticellioides[2]*, F. polyphialidicum*[2]*, F. dlaminii*[2]	E	K	B	B	EKBB
F. sporotrichioides, F. langsethiae	B	A	C	F	BACF
F. chlamydosporum, F. camptocera	B	A	C	E	BACE
F. equiseti	F	B	B	E	FBBE
F. compactum	F	H	B	B	FHBB
F. acuminatum	F	D	B	B	FDBB
F. kyushuense, F. crookwellense, F. culmorum, F. graminearum, F. flocciferum, F. lunulosporum, F. boothii[3]*, F.meridionale*[3]*, F. mesoamericanum*[3]*,F. asiaticum*[3]*, F. brasilicum*[3]*,F. cortaderiae*[3]*)*	B	A	C	B	BACB
F. poae	A	A	C	E	AACE
F. sambucinum, F. tumidum	A	A	C	A	AACA
F. robustum	A	A	C	B	AACB
F. lateritium, F. thapsinum,F. proliferatum[2]*, F. udum*[2]*, F. globosum*[2]	E	L	B	B	ELBB
F. nygamai	E	L	B	A	ELBA
F. buharicum	E	L	B	F	ELBF
F. polyphialidicum	A	B	C	F	ABCF
F. oxysporum, F. verticellioides[2]*,F. subglutinans*[2]*, F. antophilum*[2]*, F. pseudonygamai*[2]*, F. pseudocircinatum*[2]*,F. succisae*[2]*, F. bulbicola*[2]*, F. pseudoanthophilum*[2]*, F. begoniae*[2]*, F. napiform*[2]*,F. guttiform*[2]*, F. sterilihyphos*[2] *,F. lactis*[2]*, F. denticulatum*[2]	A	B	C	E	ABCE

1.*Fusarium graminearum complex*, 2. *Gibberella fujikuroi complex*, 3. *Fusarium oxysporum complex*. *.including: *F. solani, F. virguliforme, F. tucumaniae, F. phaseoli, F. brasiliensis*

**. For identifying and differentiating from other species using underlined patterns is not required.

that contain one species equally, one of them includes two species and three of them contain three species.

This study showed that using one specific enzyme could distinguish *F. nivale (Microdochium nivale), F. semitectum(F. incarnatum), F. solani* complex, *F pseudo-raminearum, F. nisikadoi, F. coeruleum* and *F. acuminatum* species (Table 4). As well by two enzymes such as; *Hha*I and MspI, F. *dimerum, F. sublunatum, F. beomiform, F. equiseti and F. compactum* could be differentiated (Table 4). As well by two enzymes such as; *Msp*I and *Taq*I, *F. sacchari, F. brevicatenulatum, F. concolor and F. ambrosium* could be distinguished. The restriction pattern of PCR –amplified rDNA of the *Fusarium* strains digested with MspI, *HhaI* and *Faq I* enzymes is shown in Figures 1-3. It shows that the bands generated corresponded to the predicted sizes.

NCBI information showed that the *Fusarium* section of *Sporotrichiella including F.poae, F. tricinctum, F. chlamydosporum* and *F. sporotrichioides / F. langsethiae* could be distinguished using the candidate enzymes (Table 4). But practically, it was seen that only *F. poae* and *F. tricinctum* could be discriminated. In order to discriminate *F. chlamydosporum* from *F. sporotrichioides* / *F. langsethiae. Mbo*I enzyme is required (Figures 4, 6). *F. compactum* from *F. equiseti* discrimination is also possible with *HhaI, Msp I* & *TaqI* enzymes (Figure 5).

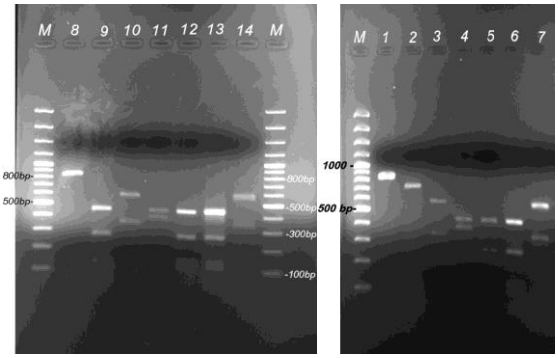

Figure 1. Restriction pattern of PCR –amplified rDNA of *Fusarium* strains digested with MspI. M: 100 bp marker. Pattern A: Lane1: *F. sporotrichioides* VTT D-72014, Lane8: *F. poae* MCR 8485. Pattern B: Lane2: *F. verticellioides* MCR 8560. Pattern C: Lane3: *F. tricinctum*, Lane10: *F. heterosporum*, Lane14: *F. tricinctum*. Pattern D: Lane7: *F. acuminatum* MCR 3231. Pattern H: Lane4: *F. compactum* MCR 2800, Lane11: *F. compactum*. Pattern L: Lane5 & 6, 12 & 13: *F. proliferatum* MCR 8549, MCR 8550 and isolates. Lane9: *F. thapsinum* MCR 8557

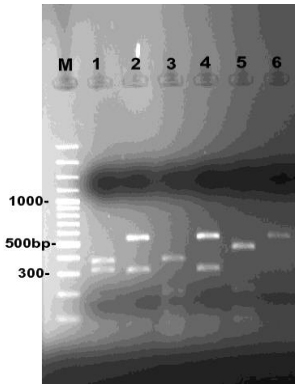

Figure 2. Restriction pattern of PCR –amplified rDNA of *Fusarium* strains digested with HhaI. M: 100 bp marker. Pattern A: Lane2& 4: *F. poae* MCR 8485, MCR 8486. *Pattern B*: Lane 6*: F. graminearum* MCR 6010. *Pattern D*: Lane 5*: F. pseudograminearum* MCR 8443*. Pattern E:* Lane1: *F. proliferatum,* MCR 8550*. Pattern F*: Lane 3*: F. acuminatum* MCR *8374*

Discussion

The restriction fragment analysis of the PCR-amplified region of rDNA from 172 isolates belonging to the 23 *Fusarium* species

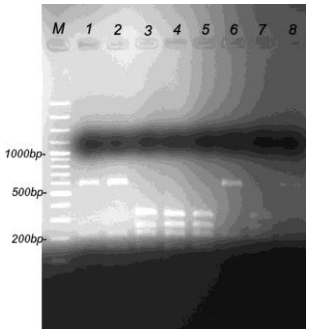

Figure 3. Restriction pattern of PCR –amplified rDNA of *Fusarium* strains digested with Faq I. M: 100 bp marker. *Pattern B:* Lane 1& 2: *F. proliferatum MCR 8549, MCR 8550.* Lane 6*: F. thapsinum* MCR 8557*,* Lane 8: *F. graminearum* MCR 6010. *Pattern E*: Lane 3*: F. sporotrichioides* MCR 0043*, Lane* 4& 5: *F. verticellioides MCR 0826, MCR 8560,* Lane 7*: F. poae* MCR 8486

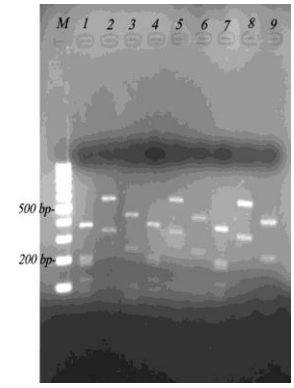

Figure 4. Gel electrophoresis of PCR –amplified rDNA of F. tricinctum strains digested with HhaI, Msp I, TaqI (FCB pattern), respectively. M: 50 bp marker. Lanes 1 to 9: isolates

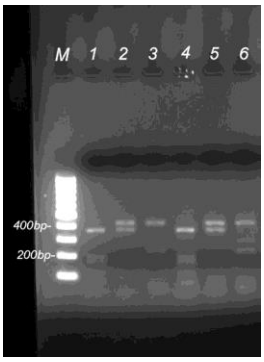

Figure 5. Gel electrophoresis of PCR –amplified rDNA of F. compactum strains digested with HhaI, Msp I & TaqI, respectively. M: 50 bp marker. Lanes 1 to 3: MCR 2800 strain, Lanes 4 to 6: isolate

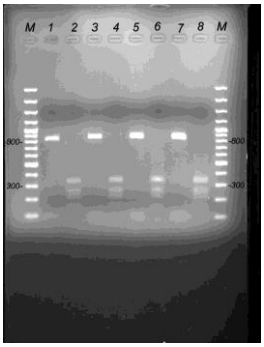

Figure 6. Gel electrophoresis of PCR –amplified rDNA of F. sporotrichioides & Fusarium cf. langsethiae strains digested with Msp I & FaqI. M: 100 bp marker. Lanes 1 to 6: F. sporotrichioides VTT D-72014, BBA 10329 & MCR 4333 strains. Lanes 7 to 8: Fusarium cf. langsethiae isolate (AE pattern)

was used. By using ITS1 and ITS4 primers, this could distinguish a limited number of *Fusarium* species in the PCR-RFLP method [20, 21, 28]. The ITS1-ITS4 or ITS4-ITS5 or ITS4-ITS5 primer pairs often amplify a 550 ± 50bp fragment from the ITS element [27] which is not lengthy enough for the PCR-RFLP technique especially in the case of *Fusarium* genus. Therefore, the reverse primer for 28S rRNA gene sequence was designed to create a larger fragment and be seen in agarose gel clearly. Besides, more patterns have been generated that can be discriminative for more species of the *Fusarium* genus.

By virtue of these findings, we conclude identifying 33 groups of *Fusarium* species and at least 22 species of them are possible through four endonuclease enzymes while the previous studies reported that via the seven enzymes can discriminate 12 *Fusarium* species (Table 3) [20, 28].

*Hha*I and *Msp*I are able to discriminate *F. equiseti* and *F.* makes a distinction on *conidia* morphology solely [2].

There are some isolates in which their new band patterns were not observed in our pattern list (Table 3). The RFLP pattern of morphology method in *F. babinda* was FLBE and also the RFLP pattern of three isolates of *F. Subglutinans* was diagnosed as -M-C. Therefore, it seems that it is possible to identify the species that are not listed in Table 4 using the proposed enzymes.

The similar patterns were observed for the several species which were distinguishable by the tease mount method. For example, the rDNA restriction pattern for *F. tricinctum*, *F. avenaceum* and *F. heterosporum* is FCBB or -CB- can be discriminated by tease mount method. *F. tricinctum* produces abundant microconidia that are napiform, oval, pyriform and citriform. *F. avenaceum* produces long and straight macroconidia but microconidiais are produced sparsely by some isolates. Moreover, *F. heterosporum* has medium length macroconidia and no microconidia. The second example is BACE pattern that can approve the existence of F. *camptoceras* using the tease mount method. According to NCBI GenBank, the *Faq*I cleavage pattern in *F. sporotrichioides* should be F (Table 3), but pattern E was observed practically (Figure 5) indicating the weakness of ITS gene in the *F. sporotrichioides* identification.

In this study, all isolates and the reference strains of *F verticellioides* had -B-E pattern whilst the GenBank information showed other patterns. The accession numbers including EU364843, EU364845 and EU364846 showed -K- B pattern while EU714404 the - D- E pattern. Consequently, it can be deduced that ITS gene in some species such as *F .verticellioides* induces interspecies' differences although it is possible that some records have been registered mistakenly

Conclusion

In conclusion, it could be concluded that using four endonucleases, namely, *HhaI*, *MspI*, *TaqI* and *FaqI* at least 22 species of *Fusarium* can be differentiated. For the

identification of the unknown *Fusarium* isolates, it is recommended to use the three enzymes, initially and *Msp*I, *Hha*I and *Taq*I, sequentially.

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Authors' contributions

R.K. designed, applied all tests and wrote manuscript, S.R. and MH.Y. managed the research and were supported financially, N.S. was scientific consultation, A.G. provided most of the reference *Fusarium* strains, F.N. applied some of the tests, V.P.edited the final manuscript.

Conflicts of interest

The authors have not supplied their declaration of conflict of interest.

Financial Disclosure

No financial interests related to the material of this manuscript have been declared.

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