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Discrimination of *Escherichia coli* Isolates Recovered from Mucosal Contents of Chicken Intestines and Different Age by Repetitive Elements Sequence-Based PCR

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ABSTRACT

Repetitive sequence-based PCR (rep-PCR) is a distinctive typing approach that is used to differentiate between bacterial strains. This method is also useful for studying bacterial diversity from different sources. In this study, four rep-PCR which are enterobacterial repetitive intergenic consensus PCR (ERIC-PCR), BOX-PCR, repetitive extragenic palindromic PCR (REP-PCR) and polytrinucleotide $(GTG)_5$-PCR were evaluated for differentiation of eighteen *Escherichia coli* isolates to correct source based on part of intestine and age. These isolates were recovered earlier from ileal and caecal mucosal contents of chickens at different age. The purpose of this study was to investigate the efficacy of four rep-PCR methods and composite of rep-PCR patterns to differentiate *E. coli* isolates to original sources of part of intestines and age based on the *D* index (discriminatory power determined based on Simpson's index of diversity calculated at similarity coefficient of 90%). The $(GTG)_5$-PCR had the highest *D* index (0.9804) for part of intestine and age factors. The similar *D* index was observed in the composite of rep-PCR patterns. The lowest *D* index was observed in ERIC- and BOX-PCR at 0.9020 and 0.8039 for part of intestine and age factors, respectively. $(GTG)_5$-PCR was also the most discriminative rep-PCR observed due to its ability to cluster 14I 3E and 14I 2X isolates, and 14C 1E and 14C 3E isolates correctly in part of intestine and age factors. It was concluded that $(GTG)_5$-PCR is a promising tool for discriminating *E. coli* isolates extracted from chicken intestines.

INTRODUCTION

Escherichia coli is a ubiquitous bacterium found as a resident of normal gut microflora in humans and animals [1-3]. Previous studies have shown that *E. coli* is one of the most abundant species observed in ilea and caeca of chickens [1, 4]. They are present as commensals or pathogens which can affect the health and dynamics of gut microflora in chickens [2, 3, 5]. For instance, *E. coli* has been effectively reduce pathogenic *Salmonella* through competitive exclusion in chickens [6]. However, the overgrowth of *E. coli* has also been reported to cause extra-intestinal infections, such as colibacillosis that caused by avian pathogenic *E. coli* (APEC) [7].

A distinctive typing approach is crucial to provide a better resolution for bacterial diversity study. Earlier profiling methods such as culture and molecular-based approaches are not able to discriminate bacterial isolates effectively up to strain

level [8]. For instance, molecular typing methods such as restriction fragment length polymorphisms (RFLPs) and 16S rRNA PCR are only able to discriminate bacterial isolates up to species level [9, 10]. Although Pulse-field gel electrophoresis (PFGE) is known as the gold standard for genotyping bacterial isolates and used in the event of an epidemic outbreak [11], it is time-consuming and labour intensive [12]. On the contrary, repetitive sequence-based PCR or rep-PCR is easy to perform, rapid and cheaper compared to other genotyping methods. Most importantly, rep-PCR has the ability to discriminate bacterial isolates at higher taxonomic resolution, by exploiting the interspersed repetitive DNA sequences of highly conserved region found throughout the genome [13].

The most common type of rep-PCR that have been used for bacterial typing are enterobacterial repetitive intergenic consensus (ERIC), BOX, repetitive extragenic palindromic (REP) and poly-trinucleotide $(GTG)_5$. These rep-PCR methods are differed based on the type of primers used to target unique sequences that are highly conserved, repetitive and naturally occurring [13, 14]. The fragments generated from rep-PCR amplification are then separated through agarose gel electrophoresis in which the unique fingerprints patterns are compared for strain differentiation based on the computer-assisted analysis.

The aim of this study was to investigate the efficacy of four rep-PCR methods of ERIC-, BOX-, REP-, and $(GTG)_5$-PCR in differentiating *E. coli* isolates that were extracted earlier from ileal and caecal mucosal contents of 7, 14, 21 and 42-day-old chickens to original sources based on part of intestine and age.

MATERIALS AND METHODS

E. coli isolates

A total of eighteen pure *E. coli* isolates were obtained from the lab that was stored in a final concentration of 15% of glycerol stocks at -80ºC. All isolates were isolated earlier from pooled ileal and caecal mucosal contents at different age of chickens and were labelled accordingly (**Table 1**).

Table 1. *E. coli* isolates obtained from mucosal contents at various part of intestine and age of chickens.

Sample	Part of Intestine	Age (Day-Old)
7I 1E	ilea	7
7I 2E	ilea	7
7I 2X	ilea	7
7I 3X	ilea	7
7C 2E	caeca	7
14I 1E	ilea	14
14I 2E	ilea	14
14I 3E	ilea	14
14I 2X	ilea	14
14C 1E	caeca	14
14C 2E	caeca	14
14C 3E	caeca	14
21I 2E	ilea	21
21C 1E	caeca	21
21C 3X	caeca	21
42I 2E	ilea	42
42I 6E	ilea	42
42C 2E	ilea	42

Rep-PCR

Genomic DNA of *E. coli* isolates was extracted by using DNeasy Blood and Tissue Kit (Qiagen, Germany) by following manufacturer instructions of DNA purification from Gram-negative bacteria. *E. coli* isolates were grown overnight in LB broth (Difco, BD, USA) at 37ºC, and then subcultured until early log phase of growth (OD_{600nm} = 0.6 – 0.8). Genomic DNA was then used as the template for four rep-PCR of ERIC, BOX, REP and $(GTG)_5$. The primers used are listed in **Table 2**. PCR amplifications were performed in a reaction mixture (25µl) containing 50-100 ng template DNA, 250 µM dNTPs, 2.5 U i-Taq Plus DNA Polymerase (iNtRON, Korea), 2 µM primer each, 1X Gitschier buffer, 10% dimethyl sulfoxide and 0.1 mg ml^{-1} bovine serum albumin using SureCycler 8800 (Agilent, USA) with an initial denaturation step at 94ºC for 2 min, followed by 30 cycles of denaturation at 94ºC for 3 sec and 92ºC for 30 sec, annealing at variable temperature for 1 min, extension at 65ºC for 8 min and a final extension step at 65ºC for 8 min. ERIC-PCR and BOX-PCR annealing temperature were set at 50ºC while REP-PCR and $(GTG)_5$-PCR was at 40ºC. Negative controls were prepared by using sterile Mili-Q water for quality check (QC) purposes. PCR products (20 µl) were resolved on 2% agarose gel electrophoresis in 1X Tris-acetate-EDTA (TAE) buffer at 120 V (4 V cm^{-1}) for 8 h in 4ºC. The 2-log DNA ladder (New England Biolabs, UK) was loaded into three wells at both ends and at the middle well of the gel. The gel was photographed under UV light in Gel Doc™ XR+ System (BioRad, USA).

Table 2. Primer sequences used for PCR amplification.

Primer	Sequence (5' – 3')
ERIC 1R	ATG TAA GCT CCT GGG GAT TCA C
ERIC 2	AAG TAA GTG ACT GGG GTG AGC G
BOXA 1R	CTA CGG CAA GGC GAC GCT GAC G
REP 1R	III ICG ICG ICA TCI GGC
REP 2I	ICG ICT TAT CIG GCC TAC
$(GTG)_5$	GTG GTG GTG GTG GTG

Rep-PCR statistical analysis

Digitised images from rep-PCR were analysed using Bionumerics Version 6.0 (Applied Maths, Belgium) based on hierarchical cluster algorithm (UPGMA; unweighted pair group method with arithmetic averages; Pearson's product moment similarity coefficient). Simpson's index of diversity is calculated at similarity coefficient of 90% for each rep-PCR and composite of rep-PCR patterns to determine their discriminatory power using the following equation [15]:

$$D = 1 - 1\frac{1}{N(N-1)}\sum_{j=1}^{s} n_j(n_j - 1)$$

where D, discriminatory index; N, total number of strains in the sample population; s, total number of types described and n_j is the number of strains belonging to the jth type.

RESULTS

Fingerprints of the four rep-PCR, ERIC, BOX, REP and $(GTG)_5$-PCR showed PCR products with the size of fragments ranging from 200 – 10 000 bp. The number of bands generated from the isolates by ERIC- (**Fig. 1**), BOX- (**Fig.** 2), REP- (**Fig. 3**) and $(GTG)_5$-PCR (**Fig. 4**) were 18-20, 23-30, 18-26 and 18-27, respectively. Out of this four rep-PCR, composite of rep-PCR patterns was generated for studying the efficacy of combining all four rep-PCR in discriminating the *E. coli* isolates. These isolates were then discriminated based on

Simpson's index of diversity and cluster analysis of part of intestine and age factors.

Based on Simpson's index of diversity at similarity coefficient of 90%, $(GTG)_5$-PCR had the highest *D* index (0.9804) for part of intestine and age factors (**Table 3**). The composite of rep-PCR patterns also had the same *D* index (0.9804). The lowest *D* index was observed in ERIC- and BOX-PCR for part of intestine and age, respectively.

Table 3. *D* index of four rep-PCR methods and composite of rep-PCR patterns in differentiating *E. coli* isolates between part of intestine and age.

Factor	*D* index				
	ERIC	BOX	REP	$(GTG)_5$	COMPOSITE
Part of intestine	0.9020	0.9412	0.9477	0.9804	0.9804
Age	0.8105	0.8039	0.9477	0.9804	0.9804

Clustering of isolates was studied in order to investigate the cluster patterns with four rep-PCR and composite of rep-PCR patterns. Based on **Table 4**, 14I 3E and 14I 2X isolates were clustered together in BOX- and $(GTG)_5$-PCR for both part of intestine and age factors. Interestingly, both isolates were consistently clustered together in all five rep-PCR in part of intestine. Both 42I 2E and 42C 2E isolates were clustered together in ERIC-, BOX- and REP-PCR for the age factor. It was also observed that 14I 3E and 14I 2X isolates; and 14C 1E and 14C 3E isolates were clustered in parts of intestine and age factors based on $(GTG)_5$-PCR.

Table 4. Clustering of *E. coli* isolates based on four rep-PCR methods and composite of rep-PCR patterns at similarity coefficient of 90%.

Factor	Cluster	REP-PCR				
		ERIC	BOX	REP	$(GTG)_5$	COMPOSITE
Part of Intestine	I	7I 1E 7I 2E 7I 2X 14I 2E	14I 3E 14I 2X	7I 3X 14I 1E 14I 3E 14I 2X	14I 3E 14I 2X	7I 1E 7I 2E
	II	7I 3X 14I 1E 14I 3E 14I 2X	7I 3X 14I 1E	NIL	14C 1E 14C 3E	7I 3X 14I 1E
	III	NIL	14C 1E 14C 2E 14C 3E 21C 1E	NIL	NIL	14I 3E 14I 2X
Age	I	42I 2E 42C 2E	42I 2E 42C 2E	42I 2E 42C 2E	14I 3E 14I 2X	14I 3E 14I 2X
	II	NIL	14I 3E 14I 2X	7I 1E 7I 2E	14C 1E 14C 3E	7I 1E 7I 2E

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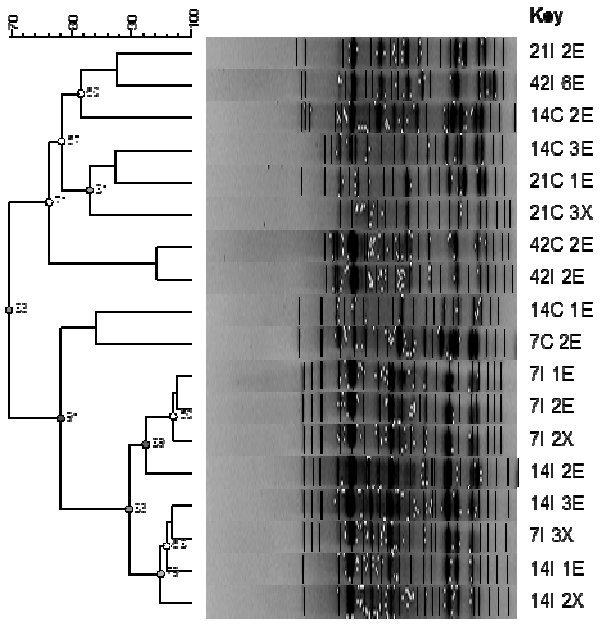

Fig. 1. Cluster analysis of ERIC-PCR fingerprints based on UPGMA of Pearson's product moment similarity coefficient set at 90%.

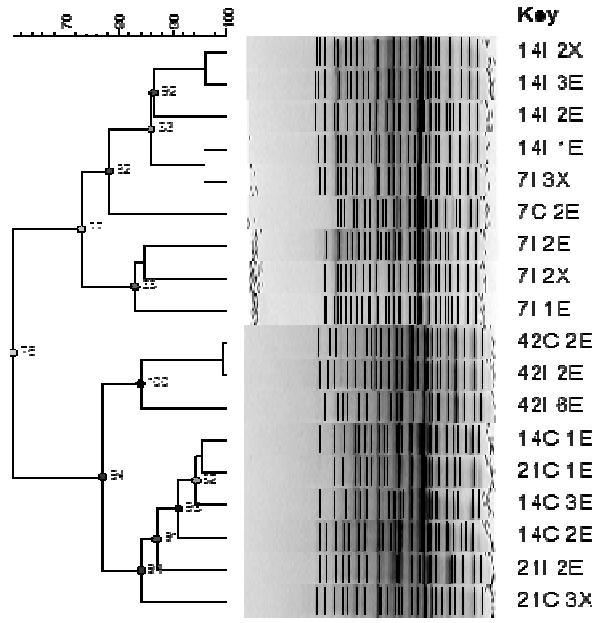

Fig. 2. Cluster analysis of BOX-PCR fingerprints based on UPGMA of Pearson's product moment similarity coefficient set at 90%.

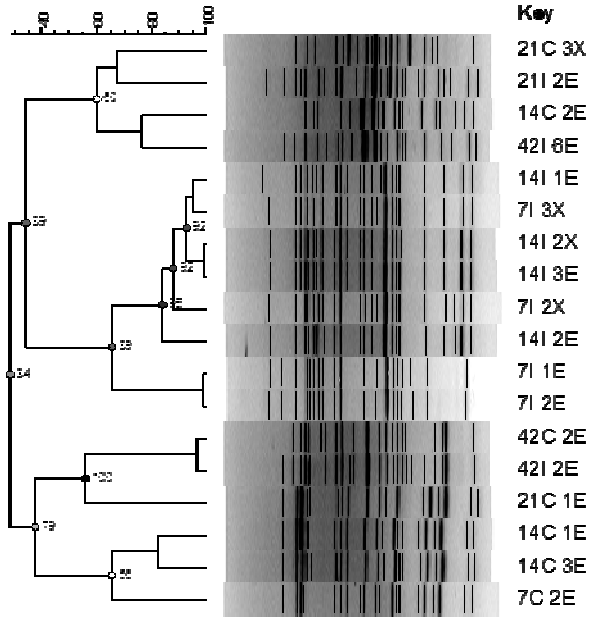

Fig. 3. Cluster analysis of REP-PCR fingerprints based on UPGMA of Pearson's product moment similarity coefficient set at 90%.

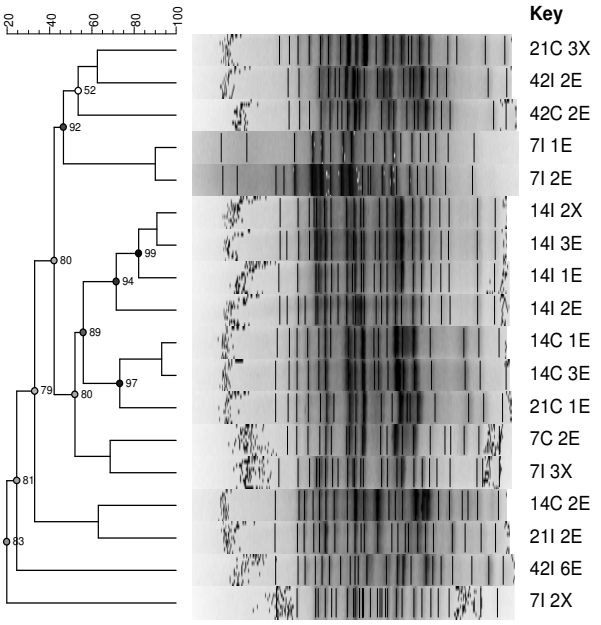

Fig. 4. Cluster analysis of (GTG)5-PCR fingerprints based on UPGMA of Pearson's product moment similarity coefficient set at 90%.

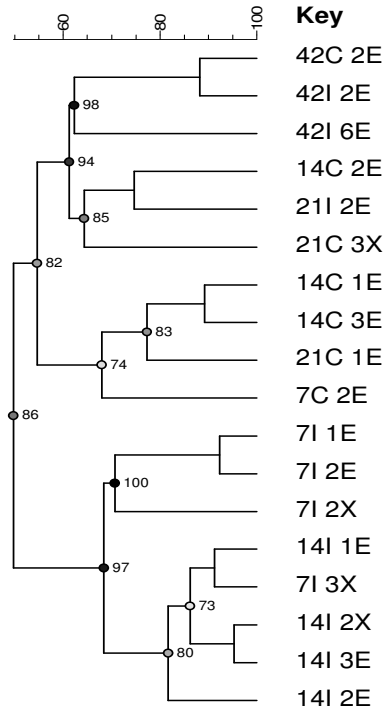

Fig. 5. Cluster analysis of composite of rep-PCR patterns fingerprints based on UPGMA of Pearson's product moment similarity coefficient set at 90%.

DISCUSSION

Four rep-PCR fingerprinting methods were utilised to differentiate *E. coli* isolates that were extracted earlier from ileal and caecal mucosal contents of chickens at the age of 7, 14, 21 and 42 days. The Simpson's index of diversity [15] was used to determine the discriminatory power (*D*) of each PCR studied, which indicated the efficacy of typing methods in differentiating between unrelated strains. In this aspect, the higher the *D* index, the higher the discriminatory power of the particular typing method to differentiate or cluster isolates into their original sources. In this study, *D* index was used to determine the rep-PCR that has better efficacy to differentiate *E. coli* isolates to original sources based on part of intestine and age. The best rep-PCR with higher *D* index was then chosen for studying the clustering of *E. coli* isolates with a better confidence.

A variety of rep-PCR methods has been used to distinguish bacterial hosts from different sources. Each method is varied by the interspersed repetitive DNA elements amplified by the specific primer(s), which varied in length and position. In the current study, $(GTG)_5$-PCR had the highest *D* index (0.9804) that was calculated for part of intestine and age factors (**Table 3**). Although the value of *D* index was the same with a composite of rep-PCR patterns, $(GTG)_5$-PCR is considered as having the highest discriminatory power instead, as a composite of rep-PCR patterns is based on average of ERIC-, BOX-, REP- and $(GTG)_5$-PCR. The $(GTG)_5$-PCR has better discriminatory power in differentiating various bacteria from different environments due to its ability to detect small genomic variations that resulted in highly variable patterns of DNA fingerprinting [16]. The $(GTG)_5$-PCR was also found to be useful to discriminate various bacteria such as *Staphylococcu*s [16], *E. coli* [17], *Salmonella* [18], *Enterococcus* [19] and

Lactobacillus [20]. This result was consistent with a previous study that shows the $(GTG)_5$-PCR was more discriminative compared to ERIC-, ERIC2-, BOX- and REP-PCR to differentiate *E. coli* isolates from aquatic environments in a study done by Mohapatra and colleague [21]. However, this was in contrast with the study reported by Dombek *et al.* [22] that shows BOX-PCR was more superior than the REP-PCR to discriminate *E. coli* from animal and human sources. In addition, Ma *et al.* [23] highlighted that BOX-PCR had a higher discriminatory power compared to both REP-PCR and $(GTG)_5$-PCR in discriminating *E. coli* from human, poultry and livestock sources in shellfish culture area. This showed that a suitable rep-PCR need to be determined first in regard to the type of isolates, source and host studied. Based on this study, it was observed that ERIC- and BOX-PCR had the lowest *D* index based on part of intestine and age factors, respectively. This suggests that they might not be suitable to discriminate *E. coli* isolates in chickens based on part of intestine and age factors. Previous study also corroborated this finding that ERIC-PCR was not suitable to distinguish *E. coli* isolates from humans and animals [24].

The bacterial isolates were investigated based on its clustering patterns to study the efficacy of rep-PCR to differentiate isolates to correct part of intestine and age. In the current study, the $(GTG)_5$-PCR was able to cluster both 14I 3E and 14I 2X; and 14C 1E and 14C 3E isolates to their original part of intestine and age (**Table 4**). This showed that 14I 3E and 14I 2X; and 14C 1E and 14C 3E isolates were correctly clustered based on their part of intestine, which was ilea and caeca, respectively and age of 14-day-old chickens, where the isolates were originally isolated. The ERIC-, BOX-, REP-, and composition of rep-PCR patterns, on the other hand, were only able to cluster the *E. coli* isolates to either their original part of intestine or age factors only (**Table 4**).

In the current study, it can be observed that the *E. coli* isolates tend to cluster with isolates from the original sources of part of intestine and age. This showed that these *E. coli* isolates could be unique to a particular part of intestine and age. This finding has also been corroborated by Joerger and Ross [25], who observed that *E. coli* isolates were unique between every chicken and also age, based on the rep-PCR study performed on *E. coli* isolated from caecal content and mucosa as chicken age. The study on gut microbial diversity in chickens also highlighted that gut microbes between ilea and caeca were distinctive, where they carried out different roles [26, 27]. This showed that the *E. coli* isolates were unique based on different part of intestines where they were isolated.

It was noted that the findings of the present study were seemed to be conflicted with previous reports. Earlier studies reported the efficacy of rep-PCR and differentiation of isolates based on dendrograms or fingerprints which are not as accurate as using discriminatory power based on Simpson's index of diversity statistical analysis [28]. The dendrograms and fingerprints discrimination methods are also based on subjective assessment, and impossible to be replicated between studies. The statistical method used for generation of fingerprints can also influence the differences between studies. It has been shown that the use of Jaccard band-based method was not effective in differentiating *E. coli* isolates compared to those of curve-based method [29]. The curve-based quantitative method includes Pearson's product-moment correlation coefficient which was used in this study. The use of curve-based method is also superior to the band-based method due to the ability to differentiate highly similar DNA fingerprints, and less sensitive to DNA concentration and background differences in gels [29]. The differences reported between these studies may also be due to different isolates studied as repetitive elements present in the bacterial genomes are highly variable between bacterial strains [28].

This study could be further clarified with some improvement. As mucosal contents of each chicken were pooled instead of studying a single sample for each chicken, it would be difficult to deduce if that particular strains of *E. coli* are unique to each host. The determination of cut-off point may need to be chosen appropriately due to many factors, such as statistical methods, diversity of strain, the pattern of electrophoresis and technical method. Similarity coefficient set at 90% in this study may be considered as too sensitive, but crucial for the sake of confidence in discriminating *E. coli* isolates and comparing PCR efficacy. However, it is advisable to choose the cut-off depending on the factors mentioned. The image resolution could be improved by using capillary electrophoresis in order to obtain a higher resolution image, thus providing high fidelity and unbiased discrimination [30].

Conclusion

In conclusion, this study demonstrated the efficacy of four rep-PCR methods of ERIC-, BOX-, REP-, $(GTG)_5$-PCR and composite of rep-PCR patterns in differentiating *E. coli* isolates that were extracted earlier from ileal and caecal mucosal contents of 7, 14, 21 and 42-day-old chickens to original sources based on part of intestine and age. The $(GTG)_5$-PCR showed the highest *D* index compared to ERIC-, BOX-, REP-PCR. This was reflected in the ability of $(GTG)_5$-PCR to differentiate *E. coli* isolates to original source based on part of intestine and age factors which cannot be achieved by other rep-PCR. Therefore, $(GTG)_5$-PCR can be promising tools for differentiating *E. coli* isolates to original source based on part of intestine and age. It was also useful for differentiating between *E. coli* isolates that were obtained from chicken intestines.

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LIST OF ABBREVIATIONS

Rep-PCR: repetitive elements sequence-based polymerase chain reaction
D: discriminatory power
APEC: avian pathogenic *E. coli*
RFLPs: restriction fragment length polymorphisms
PFGE: pulse-field gel electrophoresis
EMB: ethylene methylene blue
QC: quality check
TAE: tris-acetate-EDTA
UPGMA: Unweighted pair group method with arithmetic averages

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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