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Tropical Animal Scince Journal (Trop. Anim. Sci. J.) previously Media Peternakan is a scientific journal covering broad aspects of tropical animal sciences. Started from 2018, the title is changed from Media Peternakan in order to develop and expand the distribution as well as increase the visibility of the journal. The journal is published FOUR times a year in March, June, September, and December by Faculty of Animal Science, IPB University (Bogor Agricultural University), associated with Animal Scientist's Society of Indonesia. Tropical Animal Scince Journal is a peer reviewed journal that has been indexed and abstracted in Elsevier products (Scopus, Reaxys), Clarivate Analytics product (Emerging Sources Citation Index), Scimago Journal Rank, ASEAN Citation Index, DOAJ, Dimensions-Digital Science, CABI, EBSCO, Science and Technology Index (SINTA), Google Scholar, and other scientific databases. The journal also uses Similarity Check to prevent any suspected plagiarism in the manuscripts.

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HISTORY

Tropical Animal Science Journal (Trop. Anim. Sci. J.) (p-ISSN 2615-787X and e-ISSN 2615-790X) previously Media Peternakan (published from 1967-2017 with p-ISSN 0126-0476 and e-ISSN 2087-4634) is a scientific journal covering broad aspects of tropical animal sciences. Started from 2018, the title is changed from Media Peternakan in order to develop and expand the distribution as well as increase the visibility of the journal. The journal is published FOUR times a year in March, June, September, and December started from the year 2020 by Faculty of Animal Science, IPB University (Bogor Agricultural University), associated with Animal Scientist's Society of Indonesia.

The first edition with the new title was published in April 2018 edition (Vol 41 No 1 2018), while the previous edition (up to 2017 edition) still use Media Peternakan as the title. The online version of Tropical Animal Scince Journal could be accessed in the new website (http://journal.ipb.ac.id/index.php/tasj) and the previous editions are available in the old website (http://medpet.journal.ipb.ac.id/).

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Genetic Identification of Shiga Toxin Encoding Gene from Cases of Multidrug Resistance (MDR) *Escherichia coli* Isolated from Raw Milk

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ABSTRACT

***Escherichia coli* is one of bacteria which have resistant to three or more classes of antimicrobial agents. *E. coli* having resistant to three or more classes of antimicrobial drugs can be defined as multi-drug-resistant (MDR) bacteria. The aim of the study was to evaluate the expression of Shiga toxin gen in MDR *E. coli.* A total of 250 raw milks samples were taken from dairy farms in Kediri, Probolinggo, Pasuruan, Blitar, and Batu Region, East Java Province, Indonesia. Each sample was cultured into enrichment media Brilliant Green Bile Lactose Broth and Eosin Methyllen blue agar, then identified with TSIA agar and IMVIC biochemistry test. Antibiotic sensitivity testing was done using Kirby-Bauer disc diffusion assay on medium Mueller-Hinton agar (Oxoid, CM0337). Antibiotics disks used were 30 μg of Tetracycline (Oxoid, CT0054), 10 μg of Streptomycin (Oxoid, CT0047), 30 μg of Chloramphenicol (Oxoid, CT0013), 5 μg of Trimethoprim (Oxoid, CT0057), and 30 μg of Aztreonam (Oxoid, CT0264). Isolate showing resistance to at least 3 antibiotics disk were then continued with PCR assay to identify Shiga toxin *E. coli* (STEC) encoding *stx2* gene. The study was designed to evaluate the nucleotide analysis of STEC gene. The result showed that 6.25% (1/16) of STEC encoding gene was found in MDR *E. coli*. This report of molecular identification on the presence of STEC gene in MDR *E. coli* confirmed a wider spread of MDR *E. coli* that can threaten animal health and human health.**

Keywords: stx2 gene; MDR; Escherichia coli; raw milk; public health

INTRODUCTION

Escherichia coli which produces Shiga toxin (STEC), is defined as a group of *E. coli* which can produce a toxin called Shiga toxin (stx). There are two main types of Shiga toxins found in this STEC group. The two toxins are *stx1* and *stx2* (Hunt, 2010). According to several reports of findings in the field, *stx2* type toxin is more common and more prevalent than *stx1* toxin in *E. coli* isolates obtained from livestock feces (Tahamtan *et al*., 2010). Cattle are believed to be the main reservoir of the STEC bacteria (Hussein & Sakuma, 2005). STEC is often identified with foodborne outbreaks throughout the world with mild symptoms such as mild diarrhea to severe symptoms such as hemolytic uremic syndrome (HUS) (Noris *et al*., 2012). Foods related to STEC infection are foods without cooking processes, such as raw meat, cheese, non-pasteurized milk or raw milk, fruit and vegetable juices, and other natural ingredients (Mohammadi *et al*., 2013).

Antimicrobial resistance is a global problem, in which scientific research has been carried out and indicates that this problem has a negative impact on human and animal health (Ventola, 2015). Irrational treatment and administration of antibiotics greater than the recommended dosage in agriculture increase the incidence of antimicrobial resistance in daily life, and this condition has a great impact on the social and economic aspects (Barriere, 2014). Antibiotic resistance makes health workers lose many antibiotic choices in the treatment of bacterial diseases, causing the high potential for treatment failure in cases of clinical complications (Llor & Bjerrum, 2014). The applications of good hygiene and sanitation practices in animal farms are something that should be emphasized to prevent the distribution of MDR microbes in the populations and the transfer of MDR microbes from animals to humans.

Although antibiotic treatment is not the first choice in cases of infections caused by STEC, multidrug-resistant (MDR) STEC is a public health problem that is quite dangerous because the strain acts as a reservoir of resistant genes (Cointe *et al*., 2018). MDR STEC bacteria can easily transfer the resistant genes to the other Enterobacteriaceae family bacteria in the body of living organisms or in the environment. Some bacteria

in the gut of living organisms are friends and have the potential to transmit resistance genes to one another (Colavecchio *et al*., 2017). A number of researches have been conducted on STEC isolates from humans, but only a few have been identified from foodstuffs of animal origins. Report on the status of STEC bacterial resistance to antibiotics is also limited. Therefore, this study was conducted to identify the STEC gene, *stx2*, which is a prevalent form of MDR *E. coli* bacteria isolated from several dairy farms in East Java, Indonesia.

MATERIALS AND METHODS

The sample size in this study was 250 raw milk samples taken from the cow bulk milk of each dairy farm located in Kediri (K), Probolinggo (A), Pasuruan (G), Blitar (S), and Batu (H), East Java, Indonesia, during the period of September-December 2019. The study was conducted by purposive sampling with sample criteria of poor enclosure sanitation and dirty environment (Dairy, 2019). The number of farms calculated on average reached 1500-1800 lactation cows per location. Samples taken at each dairy farm location were 50 samples (cow bulk milk). A total of 25 mL of milk samples from each milk can be taken and placed in sterile plastics. Milk samples were put into a cool box at 4°C for transportation (Arya *et al*., 2012; Kristianingtiyas *et al*., 2020; Putra *et al*., 2019).

Each sample was cultured into enrichment media of Brilliant Green Bile Lactose Broth (Merck, 105454) and incubated at 37°C for 18-24 hours. The positive results were characterized by the change of media from green to cloudy green color and the presence of gas in Durham tube (Effendi *et al*., 2019). The positive samples were then cultured in Eosin Methylene Blue (EMB) (Merck, 101347) and incubated at 37°C for 18-24 hours (Putra *et al*., 2019). The colonies showing metallic green colors were purified and continued to the identification steps with Triple Sugar Iron Agar (TSIA) and Indol-Motility, Methyl Red, Voges Proskauer, and Citrate (IMViC) tests. The positive samples were then stored in LB Broth (Himedia, M1245) for further use in the next testing.

Antibiotic sensitivity testing was done using Kirby-Bauer disc diffusion assay on Mueller-Hinton agar medium (Oxoid, CM0337). Antibiotics disks used were 30 µg of Tetracycline (Oxoid, CT0054), 10 µg of Streptomycin (Oxoid, CT0047), 30 µg of Chloramphenicol (Oxoid, CT0013), 5 µg of Trimethoprim (Oxoid, CT0057), and 30 µg of Aztreonam (Oxoid, CT0264). Interpretation of results was conducted by measuring the diameter of the inhibitory zone formed, based on Clinical and Laboratory Standards Institutions, and the resistance to Aztreonam that is the monobactam can be referred as a presumptive extended spectrum beta lactamase (ESBL) (CLSI, 2018).

Isolate which shown resistance to at least 3 antibiotics disk was then taken from the LB Broth and transferred onto Nutrient Agar (Merck, 105450). The whole cell suspensions were made by mixing approximately 8-10 colonies from Nutrient Agar to 0.3 mL Tris-HCl containing EDTA (TE) buffer. The cell lysate was made by heating the suspension for 10 min with the boiling method. The lysates were centrifuged for 10 min at 8000 rpm to shed the cellular debris. A volume of 5 µL of the supernatant was used as a template for amplification by PCR. The specific primer used in this study was *stx2* according to Brenjchi *et al*. (2011), F: 5′- CCA TGA CAA CGG ACA GCA GTT-3′ and R: 5′- CCT GTC AAC TGA GCA CTT TG-3′.

Each PCR reaction was performed in a 25 µL amplification mixture consisting of 2.5 µL 10 X PCR buffer (500 mM KCl, 200 mM Tris HCl), 0.5 µL dNTPs (10 mM), 1 µL $MgCl_2$ (50 mM), 1.25 µL of each primer (0.5 µM), 0.2 µL of Taq DNA polymerase (5 unit/µL), and 2 µL of template. The thermocycler (Bio Rad, Hercules, CA) program was started with initial incubation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 60 sec, annealing at 52°C for 30 sec and elongation at 72°C for 60 sec, and a final extension at 72°C for 10 min. The PCR products were separated by electrophoresis in 1.5% agarose gel at 100 V for 40 min in the tris-acetate buffer, visualized by ethidium bromide staining, illuminated by UV-transilluminator, and documented by a gel documentation apparatus. DNA ladder with 100 bp was used as a marker for m-PCR assay. The expected size of products for *stx2* is 779 bp (Brenjchi *et al*., 2011).

RESULTS

A total of 250 samples were taken from several dairy farms in East Java Province, Indonesia. *E. coli* isolates were found in 176 samples of fresh cow's milk. The *E. coli* isolates were then tested using Kirby Bauer disc diffusion assay with several antibiotics, namely Tetracycline, Streptomycin, Trimethoprim, Chloramphenicol, and Aztreonam. The number of isolates that were resistant to Tetracycline, Streptomycin, Trimethoprim, Chloramphenicol, and Aztreonam was 17.05% (30/176), 14.2% (25/176), 9.66% (17/176), 7.95% (14/176), and 1.7% (3/176), respectively (Table 1).

Table 1. Multidrug resistant *Escherichia coli* from raw cow's milk samples

Location	Sample Size	Confirmed *E. coli*	Multidrug resistant	Presumptive ESBL
Kediri (K)	50	35	3	0
Probolinggo (A)	50	36	2	0
Pasuruan (G)	50	30	4	1
Blitar (S)	50	38	1	2
Batu (H)	50	37	6	0
Total	250	176	16	3

Note: Presumptive ESBL= presumptive extended spectrum beta lactamase.

A total of 16 isolates were multidrug-resistant *E. coli*, and 3 isolates were isolates of presumptive ESBL producing *E. coli*. Seventeen isolates of MDR *E. coli* and presumptive ESBL that were evaluated in this study had patterns of resistance that were almost the same to the pattern of resistance to aminoglycoside class drug, i.e., Streptomycin (Table 2 and Figure 1).

After finding *E. coli* MDR isolates that were resistant to various antibiotics and presumptive ESBL producing *E. coli,* the presence of *stx2* gene was tested by PCR method. From 17 isolates tested by PCR, there was 1 *E. coli* MDR isolates (5.88%) that was found in the sample obtained from Probolinggo (Sample Code A-26), which showed a PCR product of stx2 gene at the size of 779 bp (Figure 2). However, on Figures 3 and 4 there was no sample tested with a positive result of *stx2* gene.

DISCUSSION

Tetracycline has the highest resistance level because this antibiotic is used most often for dairy farming, followed by the other antibiotics such as those used in this study, namely the aminoglycoside (Streptomycin), macrolide (Chloramphenicol), and sulphonamide (Trimethoprim) groups (Hunter *et al*., 2010). The use of broad-spectrum antibiotics such as the tetracycline and beta-lactam classes are more common in clinical mastitis cases of dairy cows in Europe, due to their effective treatment results. For respiratory and digestive tract problems, the tetracycline and aminoglycoside groups are the first choice antibiotics, while the second choice is the macrolide group and the combination of sulfonamide-trimethoprim drugs, which have a significant

Table 2. Antimicrobial resistance of multidrug resistant (MDR) isolates

No.	Sample code	Resistant to					Multidrug resistant (MDR)	Presumptive ESBL	Positive to stx2 gene
		TE	S	W	C	ATM			
		30 µg	10 µg	5 µg	30 µg	30 µg			
1.	K-03	√	√	√	√	-	+	-	-
2.	K-04	√	√	√	√	-	+	-	-
3.	K-36	√	√	-	√	-	+	-	-
4.	A-26	√	√	√	√	-	+	-	+
5.	A-44	-	√	√	√	-	+	-	-
6.	G-12	√	√	√	-	-	+	-	-
7.	G-31	-	√	√	-	√	+	+	-
8.	G-35	√	√	√	-	-	+	-	-
9.	G-43	√	√	-	√	-	+	-	-
10.	S-25	√	-	-	√	√	+	+	-
11.	S-38	-	√	-	-	√	-	+	-
12.	H-11	√	√	√	√	-	+	-	-
13.	H-15	√	√	√	-	-	+	-	-
14.	H-28	√	√	-	√	-	+	-	-
15.	H-37	√	√	-	√	-	+	-	-
16.	H-44	√	√	-	√	-	+	-	-
17.	H-45	√	√	-	√	-	+	-	-

Note: TE= Tetracycline; S= Streptomycin; W= Trimethoprim; C= Chloramphenicol; ATM= Aztreonam; √ = positive resistant. MDR is resistant to three or more classes; ESBL is resistant to ATM; presumptive ESBL= presumptive extended spectrum beta lactamase; Kediri (K), Probolinggo (A), Pasuruan (G), Blitar (S), and Batu (H); + = positive; - = negative.

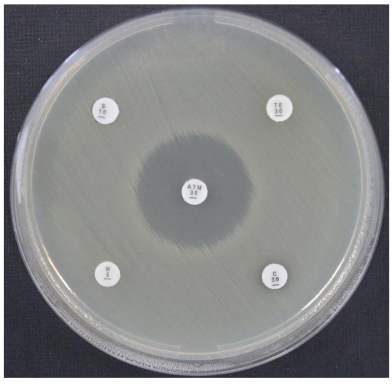

Figure 1. Multidrug resistant (MDR) *Escherichia coli* in Kirby-Bauer disk diffusion test

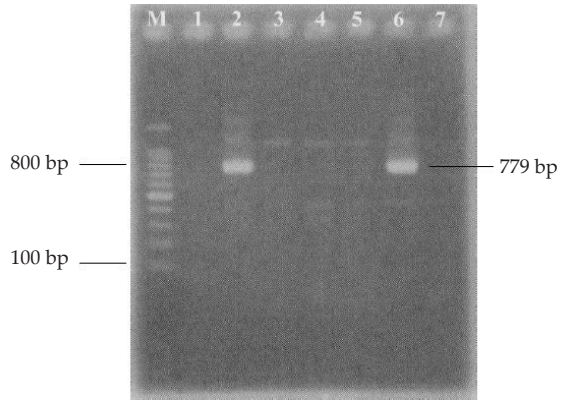

Figure 2. PCR analysis of stx2 gene on multidrug resistant (MDR) *Escherichia coli* from raw milk of cows; stx2 gene is indicated by the DNA band at 779 bp. M= marker 100 bp; 1= Negative Control; 2= Positive Control; 3= K-03; 4= K-04; 5= K-36; 6= A-26; 7= A- 44

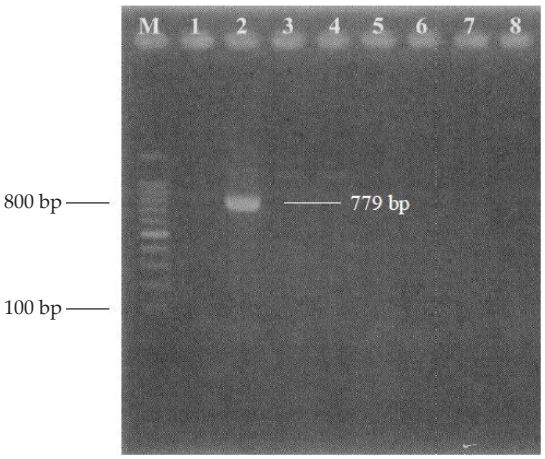

Figure 3. PCR analysis of stx2 gene on multidrug resistant (MDR) *Escherichia coli* from raw milk of cows; stx2 gene is indicated by the DNA band at 779 bp. M= marker 100 bp; 1= Negative Control; 2= Positive Control; 3= G-12; 4= G-31; 5= G-35; 6= G-43; 7= S- 25; 8= S-38.

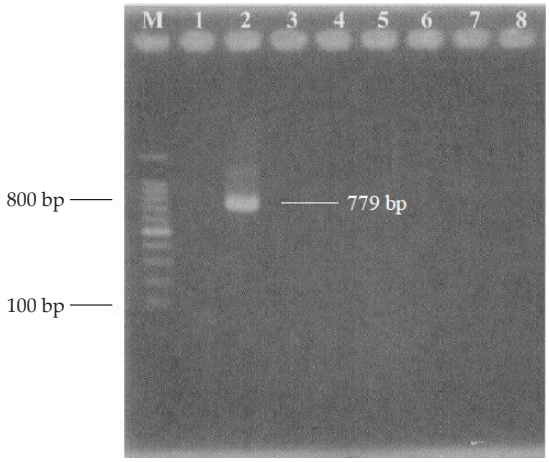

Figure 4. PCR analysis of stx2 gene on multidrug resistant (MDR) *Escherichia coli* from raw milk of cows; stx2 gene is indicated by the DNA band at 779 bp. M= marker 100 bp; 1= Negative Control; 2= Positive Control. Sample: 3= H-11; 4= H-15; 5= H-28; 6= H-37; 7= H- 44; 8= H-45.

impact on microbial activity in the rumen, and the last choice is the cephalosporin class three and four antibiotics (the sulfonamide-trimethoprim drug combination which has a significant impact on microbial activity in the rumen, and the last choice is the cephalosporin class three (Economou and Gousia, 2015).

This resistance to Streptomycin can be attributed to the effectiveness of the streptomycin drug in the treatment of mastitis in dairy cows in Indonesia. According to Riyanto *et al*. (2015), the administration of a combination of Penicillin-Streptomycin antibiotics is still considered as a good therapeutic treatment for dairy cows suffering from mastitis. Even though the level of resistance to the drug Streptomycin observed in this study is quite low (14.2%), there is a need to monitor the use of antibiotics Streptomycin in dairy cows in Indonesia.

Chloramphenicol is not approved for use in food animals in Indonesia refer to Law No. 18 of 2009, concerning Animal Husbandry and Health. The persistence of chloramphenicol resistance in *E. coli* has been observed by the other authors (Tadesse *et al.* 2012), showing that more than 90% of chloramphenicol-resistant *E. coli* isolates were concurrently resistant to tetracycline. In addition, Tadesse's data showed not only the persistence of chloramphenicol but an increasing trend of tetracycline resistance over time among animal *E. coli* isolates. These observations could be explained by co-selection of mobile resistance elements or by the unknown substrate(s) for the chloramphenicol-resistance determinants that serve as a selection pressure.

One method that is often used by various researchers to characterize organisms as Multidrug-Resistant (MDR) is based on the results of antimicrobial susceptibility testing *in vitro*, when researchers test resistance to several antimicrobial agents, classes or subclasses of antimicrobial agents (Kallen *et al*., 2010). MDR-organism is an organism that is resistant to three or more classes of antimicrobials (Magiorakos *et al*., 2012), or MDR is Gram-negative bacteria that are resistant to three or more classes of antimicrobials (Gould, 2008), as is shown in Figure 1. A review of the variability of this definition is given in a comprehensive MDR review by Falagas *et al*. (2006), which is used as a reference by some researchers that a large number of studies do not propose specific definitions for MDR, as were shown in Table 2.

The literature shows that food-producing animals represent the most important source for the inclusion of STEC in the food chain. Most infections in humans are caused by the consumption of food contaminated with STEC. Food-producing animals are important reservoirs of STEC and function as sources of food contamination. Several studies found the presence of STEC, especially in fresh milk products and their processed products (Martin & Beutin, 2011).

Shiga toxin-producing *Escherichia coli* (STEC) is a subgroup of *Escherichia coli* that are able to produce one or two strong toxins called Shiga 1 and Shiga 2 toxins (*stx1* and *stx2*), which are thought to have additional virulence factors such as intimin responsible for attaching STEC to the cells. Intestinal epithelium and causes the tight attachment and effect of lesions on the intestinal mucosa (Melton-Celsa, 2014). This bacterial pathotype is a causative agent of gastroenteritis and can be exacerbated by hemorrhagic colitis (HC) or hemolytic uremic syndrome (HUS), which is a major cause of acute kidney failure in children (Etcheverría & Padola, 2013). Foodborne diseases related to STEC have been documented throughout the world. STEC serotype O157: H7 was reported as the causative agent of a series of outbreaks that were occurred mainly in Canada (Chui *et al*., 2011), Japan (Kanayama *et al*., 2013), the United States (Mc Collum *et al*., 2012), and the United Kingdom (Adams *et al*., 2016). In Indonesia, STEC was detected in feces (8.3%) and meats (5.3%), both isolated from cattle slaughtered in Qurban festival (Ningrum et al., 2016). In addition, from samples with positive STEC tested in the slaughterhouse in Surakarta, fifteen *E coli* isolates were detected to contain stx2 gene (Goma *et al*., 2019).

Observation of raw milk in Surabaya also detected two *E. coli* (2.7%) having stx2 gene (Effendi *et al*., 2018).

One of the most debated topics in the management of STEC infections lies in the possible risks of using antibiotics in natural infections. Because antibiotics can lyse bacterial cell walls, then release Shiga toxin and cause an increase in Shiga toxin gene expression *in vivo*, generally antibiotics are not recommended for treating STEC infections (Paton & Paton, 1998). Antibiotics have long been used in animal and human medical fields for the treatment, control, and prevention of infectious diseases. However, excessive use and not according to the rules of use can have unanticipated side effects, including the development of antibiotic resistance in bacteria against modern β-lactam antibiotics (Tekiner and Özpinar, 2016) or commonly known as an Extended Spectrum β-lactamase (ESBL). Although STEC infections are not treated aggressively with antibiotic therapy, there have been many STEC isolates that are susceptible to many antibiotics, and recent reports indicate that antimicrobial resistance from STEC also continues to increase (Ahmed & Shimamoto, 2015).

Detection of STEC is labor-intensive and the total time required for strain characterization is usually 72 h. On the other hand, molecular methods are sensitive, specific, and a quick approach to detection and characterization of microbiological contaminants in food. The molecular characterization of STEC is performed by means of PCR. The study showed that among cases of MDR from *E. coli* samples, 5.88% contained STEC encoding gene. Environmental contamination, herd management, and poor milking practices are important causes of milk degradation. It has been shown that food animals are important sources of STEC's entry into the food chain (Martin and Beutin, 2011).

The finding of STEC bacteria as contaminants from raw cow milk samples in this study can be considered as important and worthy results, referring to the study of Newell and La Ragione (2018), which states that with only small amounts, STEC colonies found in food samples of animal origin can cause digestive and urogenital disorders. For this reason, housing management and good sanitation practices during the process of milking cows, as well as their storage and distribution, should be improved even better so as to keep consumers away from foodborne diseases originating from raw cow's milk or other dairy products. Therefore, hygiene practices and strict management for dairy herds, processing, and storage of milk should be adopted to avoid undesirable illness due to contaminated milk, and it is important to encourage people to pasteurize milk for consumption.

CONCLUSION

Molecular identification showed that Shiga toxin encoding gene was found in raw cow milk from several regions with cases of Multidrug-Resistance (MDR) of *Escherichia coli* in East Java, Indonesia. These results show that raw cow's milk has a potential for spreading and poses a threat to public health from *E. coli* isolates.

CONFLICT OF INTEREST

The author states that there are no conflicts of interest with financial, personal, or other relationships with other people or organizations related to the material discussed in the manuscript.

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