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Efficient Bioreduction of Sulfate from Industrial Wastewater Effluents Using *Enterobacter cloacae* emr69

Eman R. Abduallah[1], Eman M. Saad[2], Akram A. Aboseidah[1], Abdel-Hamied M. Rasmey[1*]

[1]Botany and Microbiology Department, Faculty of Science, Suez University, Suez, Egypt.

[2]Chemistry Department, Faculty of Science, Suez University, Suez, Egypt.

***Correspondence**
Corresponding author:Abdel-Hamied M. Rasmey
E-mail address: am_rasmey@yahoo.com

Abstract

This study aimed to isolate and characterize sulfur reducing bacteria from industrial wastewater and soil to remove sulfate. A total of 14 sulfate reducing bacterial (SRB) isolates were recovered from industrial wastewater and contaminated soil. Interestingly, bacterial isolate emr69 was selected as the highest sulfate reducer. Correspondingly, emr69 was characterized phenotypically and identified genotypically based on 16S rRNA gene sequencing as *Enterobacter cloacae* and deposited in Gen Bank database under accession number OR472728. The maximum sulfate reduction by *E. cloacae* emr69 against 2000 ppm (SO_4^{-2}) was 95% which obtained by adjusting the medium at pH 7 and growing the bacterium at 37°C under anaerobic conditions. The study suggests using of *E. cloacae* mr69 as a promising SRB for bioreduction of sulfate in industrial wastewater treatment.

KEYWORDS
Bioreduction, Industrial wastewater, Sulfate reducing bacteria, *Enterobacter cloacae*, Genotypic identification.

INTRODUCTION

In the last decades, sulfate-containing wastewater produced from the industrial and human activities especially the petroleum refineries represent a potential threat to the environment (Zhang *et al*., 2022). Direct discharge of sulfate contaminate wastewater into aquatic environment may cause negative effect on equilibrium of natural ecosystems (Cai *et al*., 2017). The presence of sulfate in such water over 250 ppm causes serious healthy and economic problems (Bowell *et al*., 2004).

For instance, the excessive absorption of sulfate by human body will cause several diseases, e.g., diarrhea, gastrointestinal disorders and dehydration (Man *et al*., 2014). So, sulfate ions should be eliminated from industrial wastewater before discharging into receiving waters or municipal sewerage (Bowell *et al*., 2004).

Several technologies such as chemical precipitation, membrane filtration processes and Physical adsorption have been used for wastewater treatment (Cox *et al*., 2007; Barakat, 2011; Rathoure, 2015; Morin-Crini *et al*., 2017; Wang and Zhuang, 2017) but these approaches have some disadvantages like high costs, the need for post treatment of produced water and the poisonous effect of some chemicals used for removal (Ayangbenro *et al*., 2018). Thus, a simple and cost-effective technique was required for water treatment process.

Bioremediation is one of the most suitable treatment methods because it is ecofriendly and cost-effective compared to the other traditional chemical or physical methods (Ding and Zeng, 2022). Several microorganisms can used to treat the sulfate contaminated wastewater. Most of these microorganisms are among the bacterial domain for example, sulfur reducing bacteria and sulfur oxidizing bacteria in aquatic environments (Sievert *et al*., 2007). Bioremediation strategy is pioneered than other remediation techniques for sulfate reduction since sulfate-reducing bacteria (SRB) not only utilizes sulfate as the terminal electron acceptors, but also resists various harsh environments. SRB can metabolize sulfate into low solubility sulfur compounds easy for precipitation and removal. In the biological reduction, sulfate is transformed to hydrogen sulfide as an end product (Liamleam and Annachhatre, 2007). Some strains of Enterobacter spp. had effective role in biological treatment of polluted environment with sulfate (Babu *et al*., 2014). Enterobacter spp. are considered as non-traditional sulfate reducing bacteria and used with other species for sulfate removal (de Matos *et al*., 2018).

The present study aimed to investigate the ability of *Enterobacter cloacae* emr69 isolated from industrial wastewater to remove sulfate ions from sulfate contaminated water. Moreover, the sulfate reduction process was optimized by studding the impact of several parameters like sulfate concentration, aeration, pH and temperature.

MATERIALS AND METHODS

Source of bacterial strains

Industrial wastewater samples and petroleum contaminated soil samples were collected from different locations in Suez governorate, Egypt. These samples were used as source for isolation of sulfate reducing bacteria.

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Isolation of sulfate reducing bacteria

Isolation of SRB was achieved on Postgate c medium (g/L): 0.5 KH_2PO_4, 1.0 NH_4Cl, 0.06 $MgSO_4.7H_2O$, 3.5 sodium lactate (70%), 1.0 yeast extract, 1.0 $CaSO_4$, 0.01 $FeSO_4.7H_2O$, 4.5 Na_2SO_4, 0.06 $CaC_{12}.6H_2O$, 0.3 Sodium citrate (Garcia *et al*., 2001). NaOH 0.1 M and/or 0.1 M HCl were used to adjust pH of the medium, 20 g/L agar was used when solidification is needed.

Five mL of industrial wastewater sample was mixed with 100 ml sterilized distilled water while for soil samples, 5g soil were added to 100 mL sterilized distilled water. Individually, 10 ml of the sample was then transferred to 90 mL Postgate c broth and covered by 1 cm layer paraffin oil to provide anaerobic conditions and incubated at 37ºC for 7 days. After incubation one mL culture was serially diluted until reaching the suitable dilution. One mL of each dilution was spread aseptically to sterilized Postgate c medium Petri dish and incubated at 37ºC for 48 h under anaerobic conditions (anaerobic jar). After incubation, the morphologically different bacterial colonies were then streaked on Postgate c agar for purification.

Sulfate reduction process

The recovered bacterial isolates were screened by inoculating on Postgate c broth and incubated at 37 ºC for 7 days and the bacterial isolates that produce black precipitate were selected as SRB. All recovered SRB were grown on Postgate c broth (2253.8 ppm SO_4^{-2}) at 37 ºC for 7 days under anaerobic conditions. Sulfate reduction was estimated spectrophotometrically using BaCl2method (Cha *et al*., 1999). Barium chloride solution (10% w/v) was added to the supernatant of incubated culture in 1:1ratio. The mixtures vortixed vigorously, a white precipitate was formed due to formation of $BaSO_4$ where turbidity was then read at 450 nm. A standard calibration curve was prepared with different concentrations of sulfate using 0-3mM (48 – 288 ppm) K_2SO4 solution and calculated from the following equation:

$$\%\,SR = (Rd/BC) * 100$$

Where %SR= percent of sulfate reduction, Rd= reduced sulfate and BC= blank concentration of sulfate.

Phenotypic characterization and genotypic identification of bacterial isolate emr69

The morphology of the purified colonies of bacterial isolate emr69 was recorded according to color, margin, shape and surface by naked eye on Postgate C medium. Gram staining was studied using bright field microscope. All biochemical characteristics such as lactose and glucose fermentation, starch hydrolysis, urease, H2S production, citrate, gelatin hydrolysis, MR-VP, catalase and indole tests were determined according to Bergy's manual of systematic bacteriology standard methods (Issazadeh *et al*., 2013).

After extraction of DNA, specific primers of 16SF: 5'GAGTTTGATCCTGGCTTAG-3' and 16SR: 5'-GGTTACCTTGTTACGACTT-3'were used. The 16S rRNA encoding gene was amplified by the polymerase chain reaction (PCR) from purified genomic DNA primers. The PCR amplification was carried out using Qiagen Proof-start Tag Polymerase kit (Qiagen, Hilden, Germany). Thermal profile for Cycle Sequencing PCR: an initial denaturation for 1 min at 96ºC, 25 Cycles of denaturation for10 s at 96ºC, annealing for 5 s at 50ºC, and extension for 4 min at 60ºC). After additional step of purification with CENTRI-SEP Columns (PRINCETON SEPARATIONS), DNA sequencing was applied by 3500 Genetic Analyzer, Applied Biosystems.

Sulfate reduction optimization study

The selected bacterial isolate emr69 was grown and incubated at different parameters. The effect of aeration (aerobic or anaerobic) on sulfate reduction by bacterial strain emr69 was detected. The effect of different medium pH values (4, 6, 7, 8 and 9) was studied. Also, the effect of different incubation temperatures (5, 30, 37 and 45 °C) was studied.

RESULTS

Isolation, screening and identification of sulfate reducing bacteria.

In the present study, thirty bacterial isolates from industrial wastewater samples and petroleum contaminated soil samples were isolated on Postgate c medium (3774.8 ppm of sulfate ion) under anaerobic conditions, incubation proceeded for 7 days. The screening for sulfate reducing bacteria depended on the ability of selected isolates on formation of black precipitate. 14 bacterial isolates could produce black precipitate and selected as SRB (Table 1). Isolate emr69 was selected as high efficiency sulfate reducing bacterial isolate where it reduced (92%) of 2253.8 ppm (SO_4^{-2}).

Table 1. Screening of the sulfate reduction by the selected bacterial isolates.

Isolate code	R_s	R_d	% SR
emr64	1842.5	411.3	18
emr65	1522.5	731.3	32
emr66	2115	138.8	6
emr67	547.5	1706.3	76
emr68	1667.5	586.3	26
emr69	182.5	2071.3	92
emr70	362.5	1891.3	84
emr71	700	1553.8	69
emr72	740	1513.8	67
emr73	1070	1183.8	53
emr74	457.5	1796.3	80
emr75	817.5	1436.3	64
emr76	1002.5	1251.3	56
emr77	2087.5	166.3	7

The selected SRB emr69 was Gram negative rod bacterial cells and had circular mucoid colonies with entire margin and green pigment on Postgate c medium. It was positive for glucose and lactose fermentation, gas production from glucose and lactose fermentation, gelatin hydrolysis, urease, and catalase tests. It was negative for starch hydrolysis, H_2S production, MR-VP and indole tests (Table 2).

For genetic characterization of sulfate reducing bacterial isolate emr69 16S rRNA gene sequence was PCR amplified from genomic DNA of the isolate. The 16S rRNA gene sequence of the selected bacterial isolate were submitted to National Center for Biotechnology Information (NCBI) under the accession number OR472728. To find the closely sequence, the (NCBI) Basic Local Alignment Search Tool (BLAST) was used, where the results viewed that the selected isolate emr69 belonged to family Enterobacteriaceae, genus Enterobacter and identified as *Enterobacter cloacae* emr69, the phylogenetic tree of *E. cloacae* emr69 and the related similar species was shown in Figure 1.

Table 2. Morphological and physiological characterization of the selected bacterial isolate *Enterobacter cloacae* emr69.

Characteristic	Observation
Colony colour	Light green
Colony margin	Entire
Colony surface	Mucoid
Colony shape	Circular
Gram reaction	-
Cells shape	Rod
Lactose fermentation	+
Gas from lactose	+
Glucose fermentation	+
Gas from glucose	+
Starch hydrolysis	-
H_2S production	-
Gelatine hydrolysis	+
Urease test	+
MR test	-
VP test	-
Indole test	-
Catalase test	+

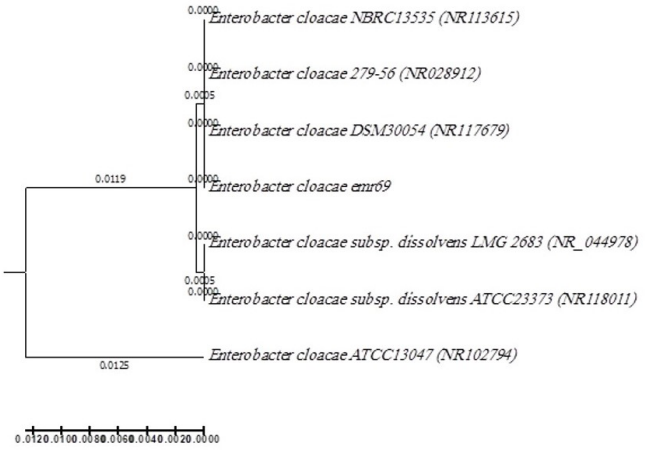

Figure 1. The neighbour-joining tree based on 16S rRNA gene sequences showing the position of the isolate *Enterobacter cloacae* emr69 and related strains.

Effect of incubation time on sulfate reduction at different concentrations

The effect of incubation time (24, 48, 96, 144, 168 and 192 hours) on sulfate reduction was illustrated in Figure 2. The results showed increased sulfate reduction with time. The highest sulfate reduction percent obtained by bacterial isolate *E. cloacae* emr69 was after incubation for 192 h at (1000 and 2000 ppm of sulfate), where it reduced sulfate by 91% when sulfate concentration was 1000 ppm and 79% when sulfate concentration was 2000 ppm. For remained concentrations 3000, 4000 and 5000ppm the percent of sulfate reduction was 57%, 61% and 43%, respectively, after incubation for 192 h.

Effect of aerobic and anaerobic conditions on sulfate reduction

The data presented in Figure 3 studied the effect of oxic conditions (aerobic and anaerobic) on sulfate reduction by *E. cloacae* emr69. Where sulfate reduction by bacterial isolate *E. cloacae* emr69 in aerobic conditions was 83% and 84% in anaerobic conditions against 2000 ppm (SO_4^{-2}).

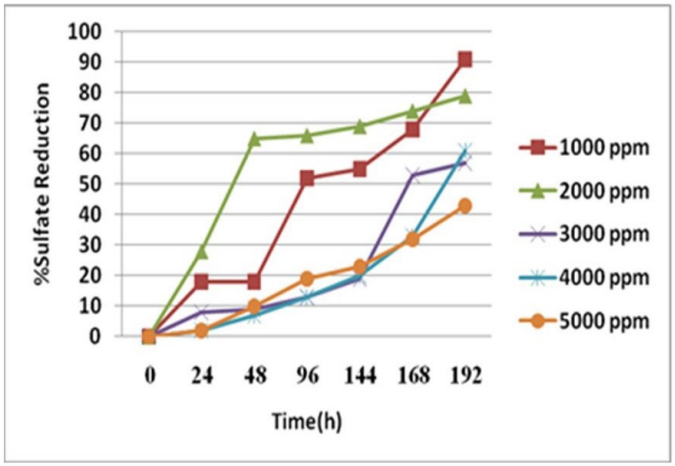

Figure 2. Effect of incubation time on sulfate reduction by *E. cloacae* emr69.

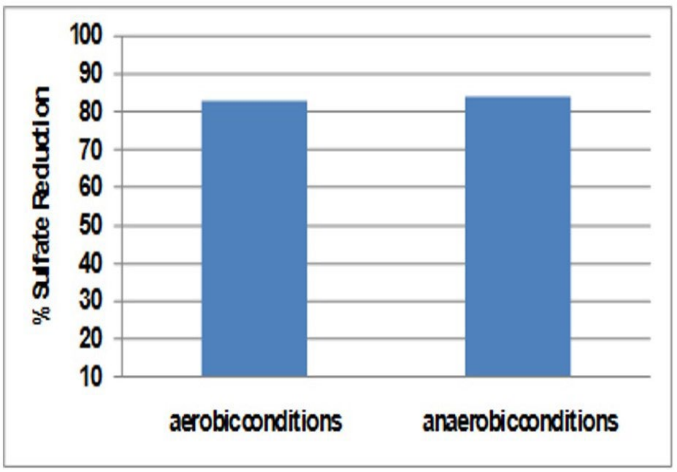

Figure 3. Effect of aerobic and anaerobic conditions on sulfate reduction by bacterial isolates *Enterobacter cloacae* emr69.

Effect of pH on sulfate reduction

The effect of pH (4, 6, 7, 8 and 9) on sulfate bioreduction against 2000 ppm (SO_4^{-2}) was illustrated in Figure 4, where the bacterial isolate had the ability to reduce sulfate at all pH rang tested. The data showed that neutral pH (6-7) was optimum for highest sulfate reduction by *E. cloacae* emr69. Acidic pH (4) and alkaline pH (8-9) showed lowest sulfate reduction by *E. cloacae* emr69.

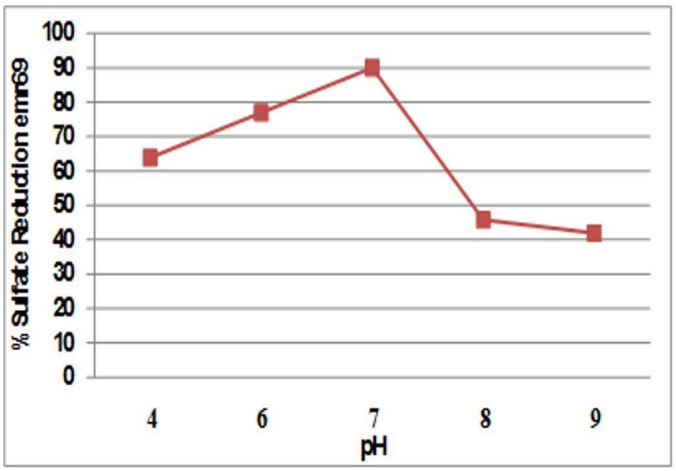

Figure 4. Effect of pH on sulfate reduction of 2000 ppm (SO4-2) Sulfate reduction by *Enterobacter cloacae* emr69.

Effect of temperature on sulfate reduction

Effect of temperature (5, 30, 37 and 45°C) on reduction of

2000 ppm (SO_4^{-2}) by *E. cloacae* emr69 has been illustrated at Figure 5. *E. cloacae* emr69 could reduce sulfate at all temperature range and the optimum temperature for sulfate reduction by bacterial isolate E cloacae emr69 was 37°C with highest sulfate reduction efficiency by 95%.

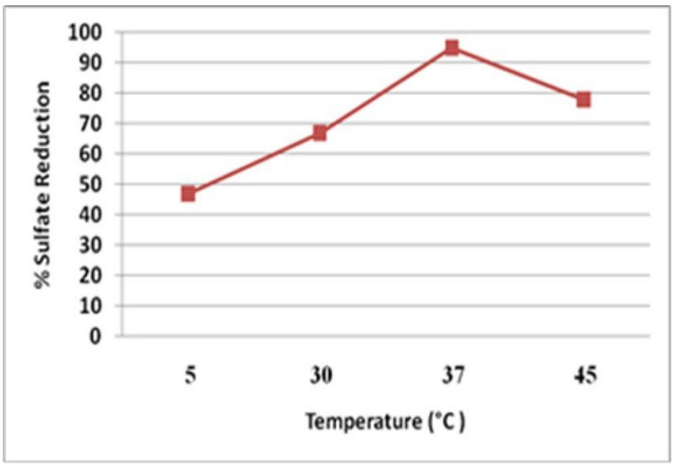

Figure 5. Effect of temperature on sulfate reduction by *Enterobacter cloacae* emr69.

DISCUSSION

Biological reduction of sulfate is broadly used for decontaminating wastewaters containing sulfate (Liamleam and Annachhatre, 2007). In this study, 14 bacterial isolates out of thirty bacterial isolates were isolated on Postgate c medium and selected sulfate reducing on ability to produce black precipitate. Virpiranta *et al.* (2021) used modified Postgate medium to observe growth of SRB by formation of black precipitate of iron sulfides. emr69 was selected as high efficiency sulfate reducing bacterial isolate where it reduced (92%) against 2253.8 ppm (SO4-2).

In a study by Najib *et al.* (2017), he used SRB isolate to remove 98% of initial sulfate concentration (2153.15 ppm of sulfate ions) in an up-flow anaerobic sludge blanket bioreactor (UASB). As well as Jiménez-Rodríguez *et al.* (2010) achieved 68.1% sulfate reduction by biological removal of sulfate from acid mine drainage. The selected bacterial isolate emr69 was identified as Gram-negative *Enterobacter cloacae*.

In a similar study, Babu *et al.* (2014) had isolated 5 bacterial strains belonged to genus Enterobacter with effective role in biological treatment of polluted environment with sulfate. Also, de Matos *et al.* (2018) reported that Enterobacter sp, Ralstonias sp, Citrobacter sp, Pantoea agglomerans, *Cupriavidus metallidurans* and *Burkholderia cepacia* were used for sulfate and arsenic removal and he referred to Enterobacter sp and Cupriavidus metallidurans as non-traditional sulfate reducing bacteria as they could produce hydrogen sulfide when grown on modified Postgate c medium.

Enterobacter cloacae considered as sulfidogenic bacteria as it produces H2S from anaerobic growth as illustrated by Duque *et al.* (2013). Palaniappan and Toleti (2016) isolated Enterobacetr cloacae and Serratia marcescens to deteriorate iron by the interaction of H2S produced by bacteria with iron in water. In a study by Rahman *et al.* (2015) has been used *Enterobacter cloacae* B2-DHA for bioremediation of Cr (VI) as Enterobacter sp were used for bioreduction and removal of Cr(VI) since they could reduce sulfate to sulfide which reacted with CrO_4^{-2} and form Cr (III) and elemental sulfur as illustrated by Sun *et al.* (2020).

The results recorded showed increased sulfate reduction by *Enterobacter cloacae* emr69 with time. This could be explained by a study by Najib *et al.* (2017) where sulfate removal exceeded 88% when initial sulfate concentration among 1250 ppm and 2875 ppm and this percent will be decreased when initial sulfate concentration increased from 3750 ppm. Sulfate reduction efficiency by *Enterobacter cloacae* emr69 decreased at 3000, 4000 and 5000 ppm. The reason for low reduction at high sulfate concentration may be due to the inhibition effect of high sulfate concentration on bacterial activity and growth as reported by Oyekola *et al.* (2010). Also, Al Zuhair *et al.* (2008) reported that the increased high levels of sulfate had negative impact on activity of SRB which had reverse impact on sulfate reduction. So, it is important to adjust sulfate concentration up to optimum level.

Sulfate reduction by *E. cloacae* emr69 against 2000 ppm (SO4-2) exceeded in anaerobic conditions than in aerobic conditions. The reason for high sulfate reduction in anaerobic conditions was accorded by Khehra *et al.* (2005) and Moosvi *et al.* (2005) where oxic conditions is favor for Enterobacter sp growth but not favorable for the yield of enzymes related to degradation. Also, Ji *et al.* (2016) illustrated that, genes related to metabolism, energy production and conversions of E sp had higher level in anaerobic conditions than aerobic. Also, anaerobic environment (anaerobic bioreactors) was recommended for best sulfate reduction by sulfate reducing bacteria (Rajeshwari *et al.*, 2000 and Chan *et al.*, 2009).

Neutral pH (6-7) was optimum for highest sulfate reduction by *E. cloacae* emr69. In a similar study by Hvitved-Jacobsen *et al.* (2013), he reported that the optimal pH range suitable for development of anaerobic bacteria was 7.6. Also, Postgate (1979) and Visser *et al.* (1996) reported that the optimal pH for sulfate reducing bacteria activity to be in the range of (7.0-8.0).

As well as Kaksonen and Puhakka (2007) demonstrated that the optimum pH for SRB growth is around 7.0. Acidic and alkaline pH showed the lowest sulfate reduction by *E. cloacae* emr69. The authors added that low pH has inhibitory effect on biological sulfate reduction. The reason for low removal at alkaline pH may be due increased toxic effect of sulfide produced. Mccartney and Olezkiewicz (1991) observed that toxic effect of sulfide increased by increasing pH. Also, it was reported by Gutierrez *et al.* (2009) that sulfate reduction and sulfide production by sulfate reducing bacteria in sewer biofilms were significantly reduced at elevated pH (8.0-9.0).

E. cloacae emr69 could reduce sulfate at all temperature range and the optimum temperature for sulfate reduction was 37°C. In a similar study by Rahman *et al.* (2015), *Enterobacter cloacae* B2-DHA was isolated and was able to grow at 20, 30, 37 and 45°C and 37°C was optimum for *E. cloacae* B2-DHA growth.

In a similar study by Singh *et al.* (2011) he studied the effect of different temperature range (30, 35, 37 and 40°C) on sulfate reduction by SRB and reported that 37°C was the optimum temperature for sulfate removal by SRB. Wang *et al.* (2022) reported that sulfate reducing bacterial activity and reduction efficiency (RE) increased by the increasing in temperature.

CONCLUSION

In the current study, a sulfate reducing bacterial strain *E. cloacae* emr69 was isolated from industrial wastewater. The optimum conditions for sulfate reduction efficiency were low oxygen conditions, at neutral pH and 37°C. The highest sulfate reduction efficiency against 2000 ppm (SO_4^{-2}) achieved in the present study was 95%. *Enterobacter cloacae* emr69 exhibited sulfate reduction capacity at high concentrations (up to 5000 ppm) of SO_4^{-2}. It is suggested that *Enterobacter cloacae* emr69 is a promising SRB for bioremediation of sulfate in industrial wastewater.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest in this

study.

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