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Combined efficacy of silver nanoparticles and commercial antibiotics on different phylogenetic groups of *Escherichia coli*

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ABSTRACT. Silver nanoparticles (Ag-NPs) can attach to flexible polymeric chains of antibiotics, hence it can be used in combination with antibiotics against resistant bacteria. In this study, minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and MBC/MIC ratio of Ag-NPs and antibiotics (gentamicin, tetracycline, erythromycin, ciprofloxacin, nalidixic acid, cefixime, cephalixin, amoxicillin, ampicillin, and penicillin) were quantified against 50 *Escherichia coli* isolates (25 human urinary tract infection and 25 avian colibacillosis). All isolates had been assigned as four phylogenetic groups A, B1, B2, and D. The results showed that the majority of the human and broiler isolates belonged to phylogenetic groups A and B2. MBC/MIC ratio of Ag-NPs in combination with antibiotics was assessed. It was found that the MIC of the majority of broiler isolates to Ag-NPs was equal to or greater than 50 µg/ml. To conclude, a combination of penicillin and ciprofloxacin with Ag-NPs exhibited profound impact against isolates, the combinations might be applicable for treating multidrug-resistant bacteria.

Keywords: *Escherichia coli*, MBC/MIC ratio, silver nanoparticles, phylotypes

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INTRODUCTION

Extra-intestinal pathogenic *Escherichia coli* (ExPEC) causes a variety of diseases outside the intestine, including urinary tract infections (UTIs) (Skjot-Rasmussen, et al. 2012), meningitis, sepsis, abdominal infections, osteomyelitis, cellulitis, and colibacillosis in poultry (Johnson, et al. 2007). *E. coli* has been characterized in four major phylogenetic groups including A, B1, B2, and D. Most commensal *E. coli* strains belong to phylogenetic groups A and B1 whereas the most virulent strains belong to groups B2 and D (Hussain, et al. 2012; Kazemnia, et al. 2014). Generally, human uropathogenic *E. coli* (UPEC) might be transferred from food chains (Singer 2015). A study indicates that retail meat contaminated with ExPEC strains, were associated with the antimicrobial-resistant *E. coli* causing urinary UTIs (Walk, et al. 2007). The application of antibiotics in the poultry industry is a risk factor for the emergence of antimicrobial resistant bacteria (Furtula, et al. 2010). Since multidrug resistant (MDR) bacteria is increasing, then, it requires to be considered as a major public health threat. The increase of MDR bacteria has led to find and develop a novel generation of safe antimicrobials to control those microorganisms. Because of their antimicrobial properties (Kim, et al. 2007), inorganic nanomaterials such as silver nanoparticles (Ag-NPs) are widely used in medical and consumer products, including antiseptic agents, medical devices, water purification systems, food packaging, and health care products (Park, et al. 2010). Mechanisms by which Ag-NPs exert their antibacterial effects to overcome bacteria are as follows: penetrating to bacterial cell which alters transportation of electrolytes and metabolites (Ansari, et al. 2014), and affects membrane-bound enzymes leading to disruption of ATP production; destroying the stability of LPS leading to an increase in the permeability of outer membrane; damaging vital enzymes, proteins, and DNA as a result of binding of Ag-NPs with them (Yang, et al. 2012; You, et al. 2012), owing to the fact that Ag-NPs have a great affinity to react with sulfur- or phosphorus-containing compounds (Silambarasan and Jayanthi 2013; Tamboli, et al. 2012); and generating reactive oxygen species which leads to death of bacteria (Xu, et al. 2012; You, et al. 2012). It has been reported that combination of antibiotics and Ag-NPs may act as an antibiotic carrier, facilitating the approaching of hydrophilic antibiotics to bacterial surface (Ghosh, et al. 2012), and then nano-silver drug carriers may help them to cross into bacteria, causing more damage to

the cell. Additionally, Ag-NPs may share their mechanism of actions with antibiotics to cope with resistant bacteria. Moreover, regarding different modes of actions of antibiotics and Ag-NPs, it can be expected if a bacterium exhibits resistance to one agent, the other agent may overcome the bacterium with different mechanisms of action. Ag-NPs have a very lower natural tendency to induce microbial resistance than do antibiotics (Chudasama, et al. 2010). The aim of the present study was to investigate the effects of the simultaneous application of Ag-NPs and commonly used antibiotics on *E. coli* of different phylogenetic groups.

MATERIALS AND METHODS

Bacterial Strains

A total of 50 *E. coli* strains (25 from UTIs and 25 from broiler colibacillosis) were included in this study. All strains (tables 1 and 2) had previously been classified using triplex PCR as proposed by Clermont *et al.* (Clermont, et al. 2000) in our previous work (Kazemnia, et al. 2014). The results of phylogenetic typing of strains are in tables 1 and 2.

Susceptibility of *E. coli* isolates to Ag-NPs

Commercially manufactured 20 nm Ag-NPs (US Research Nanomaterials, Inc., Houston, USA) was used to treat the isolates. Firstly, all *E. coli* isolates were grown overnight and washed twice, then cells were re-suspended in Muller-Hinton broth until their concentration reached 10^8 CFU/mL. The concentration was evaluated by optical density at 600 nm ($OD_{600} \sim 0.1-0.13$). Microdilution method was employed to examine the susceptibility of isolates to different concentrations of Ag-NPs. An amount of 100 μ L of each overnight culture and 800 μ L of fresh Mueller-Hinton broth were added to each well in a 48-well microplate. To treat each strain with a final concentration of Ag-NPs (2.5, 5, 10, 20, 30, 40, 50, 100 μ g/mL), 100 μ L of stock solutions of prepared Ag-NPs (25, 50, 100, 200, 300, 400, 500, 1000 μ g/mL) was added to each well. The microplates were then incubated at 37 °C for 24 h. The MIC was determined by measuring the optical density of cultured bacteria using a spectrophotometer at 600 nm. (CLSI 2012). Five microliters of each well was plated on Luria-Bertani agar and incubated further at 37 °C in order to determine the minimum bactericidal concentration (MBC) (Ayala-Núñez, et al. 2009).

Table 1. MIC, MBC and MBC/MIC ratio of SNP and antibiotics alone and in combination against different urinary phylotypes of *E.coli*.

Samples	Phylotypes	SNP		Gentamicin			Penicillin		Ampicillin		Amoxicillin		Tetracycline	
		MIC	MBC	MIC	MBC	MBC/MIC ratio in combination with 5µg/ml SNP	MIC	MBC/MIC ratio in combination with 5µg/ml SNP	MIC	MBC/MIC ratio in combination with 5µg/ml SNP	MIC	MBC/MIC ratio in combination with 5µg/ml SNP	MIC	MBC/MIC ratio in combination with 5µg/ml SNP
1	A	25	25	25	25	1	75	100	**	25	50	1	100 *	1.5
2	B2	25	25	*	*	**	**	**	**	**	**	**	**	1
3	A	50	75	*	*	**	**	**	**	**	**	**	100 *	1
4	D	50	50	25	25	1	75	100	**	50	75	1	50	100
5	D	75	100	50	50	1	**	**	**	**	**	**	**	1
6	B2	100	100	25	25	1	**	**	**	**	**	**	25	100
7	B2	50	50	25	25	1	**	**	**	**	**	**	**	**
8	D	50	75	*	*	1	**	**	**	**	**	**	**	**
9	D	75	100	50	50	1	**	**	**	**	**	**	**	**
10	A	25	25	25	25	1	**	**	25	50	1	**	25 *	75
11	D	75	75	100	*	1	**	**	50	100	1	**	**	**
12	A	50	50	25	25	1	100 *	**	25	75	3	25	50	1
13	B2	100	100	*	*	2	**	**	**	**	**	**	**	1.33
14	A	25	25	75	*	2	**	**	**	**	**	**	**	1
15	D	100	100	25	25	1	**	**	**	**	**	**	**	**
16	A	100	100	*	*	1	**	**	**	**	**	**	25 *	75
17	B2	*	*	*	*	**	**	**	**	**	**	**	**	**
18	B2	75	75	50	50	1	**	**	**	**	**	**	**	1
19	B2	75	75	25	25	1	100 *	**	50	75	1	25	50	1
20	B2	75	100	75	75	1	100 *	**	25	100	1	25	100	1
21	B2	100	100	50	50	1	100 *	**	50	100	1	25	100	1
22	B2	100	100	50	50	1	**	**	**	**	**	**	**	**
23	D	75	75	50	50	1	**	**	**	**	**	**	**	1.33
24	A	100	100	50	50	1	100 *	**	25	100	1	25	50	1
25	A	75	100	50	50	3	100 *	**	25	100	1	25	50	1

Table 1 (continued)

Samples	Phylotypes	SNP		Gentamicin			Penicillin		Ampicillin		Amoxicillin		Tetracycline	
		MIC	MBC	MIC	MBC	MBC/MIC ratio in combination with 5µg/ml SNP	MIC	MBC/MIC ratio in combination with 5µg/ml SNP	MIC	MBC/MIC ratio in combination with 5µg/ml SNP	MIC	MBC/MIC ratio in combination with 5µg/ml SNP	MIC	MBC/MIC ratio in combination with 5µg/ml SNP
1	A	25	25	12.5	25	6	**	1.33	100 *	1	25	50	**	**
2	B2	25	25	12.5	25	1	**	**	**	**	100 *	2	**	**
3	A	50	75	*	*	**	**	**	**	**	**	**	**	**
4	D	50	50	6	50	1	**	8.33	50	100	8.33	25	50	*
5	D	75	100	12.5	50	1	**	**	**	4.17	**	**	**	**
6	B2	100	100	25	*	1	50	75	2	**	**	50	50	8.33
7	B2	50	50	75	100	25	**	**	**	**	50	50	2	**
8	D	50	75	*	*	**	**	**	**	**	**	**	**	**
9	D	75	100	*	*	1	**	**	**	**	50	75	4.1	**
10	A	25	25	100	*	1	25	50	8.3	50	100	4.17	25	50
11	D	75	75	*	*	**	**	**	**	**	50	50	8.33	**
12	A	50	50	100	*	1	**	8.3	**	2	25	50	1	75 *
13	B2	100	100	*	*	**	**	**	**	**	**	**	**	**
14	A	25	25	*	*	**	**	**	**	**	**	**	**	**
15	D	100	100	12.5	*	25	75	100	4.17	**	**	**	**	**
16	A	100	100	*	*	**	**	**	**	**	**	**	**	**
17	B2	*	*	*	*	**	**	**	**	**	**	**	**	**
18	B2	75	75	*	*	**	**	**	**	**	**	**	**	**
19	B2	75	75	25	50	1	50	75	25	50	100	**	25	50
20	B2	75	100	12.5	25	1	50	75	25	50	100	**	50	50
21	B2	100	100	12.5	25	1	50	75	25	50	100	**	25	50
22	B2	100	100	*	*	**	**	**	**	**	**	**	**	**
23	D	75	75	25	75	1	**	8.33	50	100	**	25	25	2
24	A	100	100	6	25	1	50	75	25	50	100	1	12.5	75
25	A	75	100	6	25	1	50	100	25	100 *	**	25	75	2

* refers to >100; ** refers to not determined; SNP refers to Ag-NPs.

Table 2. MIC, MBC and MBC/MIC ratio of SNP and antibiotics alone and in combination against different colibacillosis phylotypes of *E.coli*.

Samples	Phylotypes	SNP		Gentamicin			Penicillin		Ampicillin		Amoxicillin		Tetracycline	
		MIC	MBC	MIC	MBC	MBC/MIC ratio in combination with 5µg/ml SNP	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
1	B2	50	75	25	50	3	**	**	25	100	50	100	25	50
2	B2	100	100	25	50	1	**	**	25	100	50	100	25	75
3	A	50	75	25	50	3	**	**	50	100	50	*	25	*
4	B1	50	50	50	75	12	**	**	*	*	*	*	*	*
5	A	1	25	25	25	1	**	**	25	100	50	100	25	75
6	B1	75	75	25	50	6	**	**	*	*	*	*	*	*
7	D	50	100	12	25	3	**	**	50	*	75	*	25	50
8	B1	25	25	25	25	1	**	**	*	*	75	*	*	*
9	B2	75	75	25	50	1	**	**	50	*	50	*	25	100
10	B2	50	50	12	25	1	**	**	50	*	50	100	100	*
11	B2	75	75	25	50	3	**	**	75	*	100	*	25	50
12	A	50	50	25	25	1	**	**	*	*	*	*	*	*
13	A	3	25	12	25	1	100	*	50	*	50	100	4	*
14	B2	1	25	12	25	1	100	*	75	*	100	*	25	75
15	A	50	100	25	50	3	**	**	75	*	*	4	*	*
16	A	50	50	25	50	3	**	**	*	*	75	100	*	*
17	A	50	50	12	25	1	**	**	50	*	*	4	*	*
18	D	50	50	25	50	3	**	**	50	*	25	100	*	*
19	D	50	50	12	25	1	**	**	50	100	100	*	*	2
20	D	50	50	12	25	3	**	**	75	*	*	4	*	*
21	B2	50	75	12	25	3	**	**	*	*	75	*	25	100
22	B1	25	25	25	50	3	**	**	100	*	*	3	*	*
23	A	25	25	12	25	3	**	**	*	*	75	*	*	*
24	A	12	25	12	25	1	**	**	75	*	75	100	25	50
25	D	50	75	25	50	2	**	**	75	100	100	*	*	*

Table 2 (continued)

Samples	Phylotypes	SNP		Gentamicin			Penicillin		Ampicillin		Amoxicillin		Tetracycline	
		MIC	MBC	MIC	MBC	MBC/MIC ratio in combination with 5µg/ml SNP	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
1	B2	50	75	75	*	100	**	**	50	*	100	*	*	*
2	B2	100	100	50	*	100	**	**	100	*	25	100	50	100
3	A	50	75	*	*	**	**	**	*	*	25	100	*	*
4	B1	50	50	*	*	4	**	**	100	*	*	*	*	*
5	A	1	25	50	70	3	100	*	33.3	50	100	25	25	100
6	B1	75	75	100	*	8.3	**	**	100	*	100	*	*	*
7	D	50	100	50	*	50	**	**	100	*	25	50	75	*
8	B1	25	25	50	*	16.7	**	**	100	*	*	*	*	*
9	B2	75	75	50	*	75	**	**	*	*	75	*	50	100
10	B2	50	50	50	*	75	**	**	100	*	100	*	*	*
11	B2	75	75	75	*	75	**	**	100	*	50	*	75	*
12	A	50	50	50	*	75	**	**	100	*	*	*	*	*
13	A	3	25	*	*	6.25	**	**	*	*	25	50	75	*
14	B2	1	25	75	*	50	75	*	100	*	25	50	25	*
15	A	50	100	50	*	6.25	**	**	*	*	25	50	50	*
16	A	50	50	75	*	16.7	**	**	*	*	25	50	50	*
17	A	50	50	50	*	16.7	**	**	*	*	25	50	50	*
18	D	50	50	50	*	75	**	**	*	*	50	100	50	*
19	D	50	50	100	*	75	**	**	*	*	25	100	50	*
20	D	50	50	50	*	100	**	**	*	*	50	100	50	*
21	B2	50	75	50	*	33.3	**	**	100	*	12	100	25	*
22	B1	25	25	25	50	12	100	*	100	*	50	*	50	*
23	A	25	25	50	*	33.3	**	**	100	*	25	50	75	*
24	A	12	25	50	75	8.3	**	**	*	*	25	75	*	*
25	D	50	75	*	*	**	**	**	*	*	*	*	*	*

* refers to >100; ** refers to not determined; SNP refers to Ag-NPs.

Susceptibility of *E. coli* isolates to antibiotics

E. coli strains were tested for antimicrobial susceptibility using the same procedure described for susceptibility testing of isolates for Ag-NPs. The antibiotics used in this essay belonging to six different classes of antibiotics: aminoglycosides (gentamicin); tetracyclines (tetracycline); macrolides (erythromycin); quinolones (ciprofloxacin and nalidixic acid); cephalosporins (cefixime and cephalixin); penicillins (amoxicillin, ampicillin, and penicillin) (Sigma-Aldrich, St. Louis, MO, USA). Stock solutions of each antibiotic were prepared with concentrations of 15.6, 31.25, 62.5, 125, 250, 500, 750, 1000 mg/mL. Each strain was then treated with a final concentration of 1.56, 3.125, 6.25, 12.5, 25, 50, 75, 100 mg/mL of each antibiotic in a 48-well microplate.

Susceptibility of *E. coli* isolates to Ag-NPs in combination with antibiotics

For determination of MIC and MBC of each isolate, the sub-inhibitory concentration of 5 µg/mL of Ag-NPs in combination with each of antibiotics was separately applied to each of the strains to examine MIC and MBC of combined agents. The interaction of antibiotics and Ag-NPs were evaluated using 5 µg/mL of Ag-NPs and 1.56, 3.125, 6.25, 12.5, 25, 50, 75, 100 mg/mL of each antibiotic to determine MIC and MBC. The procedure was performed as described previously (CLSI 2012).

Statistical analysis

All experiments were carried out at least in three replicates and obtained data were analyzed in the SPSS (Version 21, SPSS Inc., Chicago, IL).

RESULTS

MIC and MBC

The MIC and MBC patterns of 50 strains to 10 antibacterial agents and Ag-NPs are shown in tables 1 and 2. According to the MIC and MBC results, colibacillosis isolates exhibited the highest rates of resistance to penicillin and the lowest resistance to gentamycin. It was found that the MIC of the majority of isolates from broilers to Ag-NPs was equal to or greater than 50 µg/ml. Multi-drug resistance of *E. coli* isolates from human was higher than *E. coli* isolates from broilers. Statistical analysis demonstrated that, among colibacillosis isolates, bactericidal effect of 5 µg/ml in combination with penicillin, gentamycin, cephalixin, amoxicillin, erythromycin, tetracycline,

cefixime, ciprofloxacin, nalidixic acid, and ampicillin was 100, 92, 80, 33.3, 33.3, 30.8, 10, 8.7, 0 and 0, respectively. The effect of simultaneous application of Ag-NPs and antibiotics in 34.8% of colibacillosis strains was bactericidal and in 65.2% was bacteriostatic. Maximum bactericidal effects of combined agents on the broiler strains belonged to genotypes B1, D, B2 and A calculated as 46.7, 35.7, 34 and 31.4 % respectively. The bactericidal effect of combination of 5 µg/ml of Ag-NPs with each of antibiotics (ciprofloxacin, cefixime, erythromycin, tetracycline, gentamycin, nalidixic acid, ampicillin, and amoxicillin) in the case of human strains were 81.3, 60, 50, 44.8, 22, 16.7, 10, and 9%, respectively. The effect of the combination of 5 µg/ml of Ag-NPs with antibiotics in 70.4% of strains from human was bactericidal, and in 29.6% of strains was bacteriostatic in total. The maximum bactericidal effect of combined agents against human strains belonged to genotypes of A, B2, and D calculated as 75, 70, and 63% respectively.

DISCUSSION

There is a need for a new generation of antibiotics to overcome drug-resistant bacteria because of the rapid development of antimicrobial resistance (Ansari, et al. 2013). According to previous studies, there is some evidence proving that Ag-NPs have potent antimicrobial effects individually (Ansari, et al. 2014; Radzig, et al. 2013) and show synergistic activity in combination with antibiotics (Dhas, et al. 2013; Lavanya, et al. 2013). As stated in some works, whether an agent by itself or in combination with the other agents is considered bacteriostatic when this ratio is equal to or greater than 16, and if this ratio is less than or equal to 4, the agent represents bactericidal manner (Das, et al. 2016; Prema, et al. 2017). In this study, an antibacterial performances using microdilution method against isolates elucidated that combination of a penicillin (in colibacillosis strains) and ciprofloxacin (in UTI strains) with Ag-NPs exhibited profound impact and could be used for coping with multidrug-resistant *E. coli*. According to the results, most of the combined agents used in this study had bactericidal effects. Gentamicin plus 5 µg/ml offered itself to be a promising bactericidal compound against MDR *E. coli*. The results obtained from this study are consistent with that of other researches. Indeed, gentamycin plus Ag-NPs in several studies demonstrated enhanced or even synergistic antibacterial effect against *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*,

Streptococcus agalactiae, *Streptococcus mutans*, *Bacillus cereus*, *Bacillus subtilis*, *Escherichia coli*, *Escherichia fergusonii*, *Shigella flexneri*, *Klebsiella pneumoniae*, *Salmonella typhi*, *Enterococcus faecalis*, *Aeromonas hydrophila*, *Vibrio parahaemolyticus*, *Edwardsiella tarda* and *Pseudomonas aeruginosa* (Barapatre, et al. 2016; Chhibber, et al. 2017; Ebrahimi, et al. 2017; Jamaran and Zarif 2016; Katva, et al. 2017; Mohamed, et al. 2017; Panacek, et al. 2015; Satapathy, et al. 2017; Verma, et al. 2017; Wang, et al. 2016). Moreover, in a good agreement with reported data in the literature, penicillin (Ebrahimi, et al. 2017; Panacek, et al. 2015) in contrary to some studies (Deng, et al. 2016; Natan and Banin 2017; Wang, et al. 2016) and ciprofloxacin in the case of human strains (Mohamed, et al. 2017; Yallappa, et al. 2015) in accord with our data (Panacek, et al. 2015) showed significant increase in antibacterial activities when conjugated with Ag-NPs. Furthermore, tetracycline (Deng, et al. 2016; Ebrahimi, et al. 2017; Natan and Banin 2017), amoxicillin (Mohamed, et al. 2017), cephalosporin (Refat, et al. 2017), erythromycin (Yallappa, et al. 2015) showed partially increase in antibacterial activities in the presence of Ag-NPs. On the other hand, to the contrary to our study, ampicillin (Satapathy, et al. 2017), nalidixic acid (Tawfeeq, et al. 2017) have been mentioned to have a synergistic effect. This might be attributed to the joint mechanism of action between antibiotic and Ag-NPs (Wang, et al. 2016). Combination may trigger an enhanced membrane permeability and/or reactive oxygen species production. However, little is known about the interaction between Ag-NPs and antibiotic, and also the presence of resistant genes to Ag-NPs. The difference in the behavior of the combination of Ag-NPs with antibiotics discussed in the literature could be due to the presence of resistance genes to Ag⁺, applied doses, the size, and shape of Ag-NPs, and the mechanisms of resistance to Ag-NPs in bacteria. The antibacterial capacity of Ag-NPs is dependent on the size, dose, and the shape of Ag-NPs (Majeed, et al. 2016; Prema, et al. 2017; Zheng, et al. 2018). The Ag-NPs in small size reveal higher antibacterial performance (Zheng, et al. 2018). The mechanisms by which bacterial cells exert resistance to Ag⁺ are as follows: utilizing Ag⁺ ATPase efflux pumps; multiple antibiotic resistance genes; *ybdE*, *ylcD*, *ylcC*, *ylcB*, *ylcA*, *ybcZ* genes (Nagy, et al. 2011; Silver, et al. 2006); some periplasmic Ag⁺ binding proteins (Muhling, et al. 2009; Silver, et al. 2006); plasmids carrying metal resistance genes (Silver, et al. 2006). However, bacterial resistance to Ag-NPs has

not been proven yet.

The MBC/MIC ratio has been used to consider whether an agent is a bactericide or bacteriostatic. Conversely, there were significant differences in bacteriostatic and bactericidal manners of combined agents among the phylotypes of human and broiler strains. The administration of 5µ/mL of Ag-NPs combined with antibiotics against the phylotypes A, B2, and D in the case of human strains exerted bactericidal action, whereas in the case of broiler strains it was bacteriostatic manner. The authors speculate that this effect is probably due to the tolerance (Das, et al. 2016) of broiler strains against Ag⁺ and/or Ag-NPs. Since these differences among phylotypes of *E. coli* have not been studied yet, further studies are required.

CONCLUSION

The results of this study suggest that Ag-NPs combined with antibiotics exhibit excellent bactericidal effect towards drug resistant *E. coli*. Nevertheless, one of the profound concerns despite the benefits of Ag-NPs is now the toxicity of Ag-NPs on mammalian cells, which needs to be investigated further. To the best of our knowledge, it is the first report that of MBC/MIC ratio of simultaneous application of commercially prescribed antibiotic and Ag-NPs against clinical strains.

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CONFLICT OF INTEREST

There are no conflicts of interest.

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