

Full Length Research Paper

Incidence and antimicrobial susceptibility pattern of extended-spectrum- β -lactamase-producing *Escherichia coli* isolated from retail imported mackerel fish

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During the past few years, extended-spectrum β -lactamase (ESBL)-producing *Escherichia coli* and other species of Enterobacteriaceae have become a matter of great concern in human and veterinary medicine. Several studies in recent years documented the prevalence and occurrence of ESBL-producing Enterobacteriaceae in food products such as meat, poultry and raw milk; therefore in this pilot study we examined imported raw frozen mackerel fish to determine the incidence of ESBL-producing *E. coli* from Eastern Province of Saudi Arabia. From January to March, 2012, 45 samples were purchased from various supermarkets of this region and examined for the presence of ESBL-producing *E. coli* using ChromID ESBL agar plates and further confirmed by PCR amplification. Out of 45 mackerel fish samples, 23 (51.1%) were found to be positive for ESBL-producing *E. coli* and yielded 60 isolates. The higher rate of resistance was found to be with ampicillin (100%), piperacillin (96.7%), cefotaxime (93.3%), ceftriaxone (93.3%), tetracycline (53.3%), nalidixic acid (40%) and trimethoprim (30%). The least rate of resistance was recorded among chloramphenicol (15%), ciprofloxacin (15%), noroxin (11.7%) and nitrofurantoin (5%). All the 60 isolates in this study were found susceptible to amikacin, aztreonam, cefepime, ertapenem, gentamicin and kanamycin. Further characterization by PCR revealed that 49 (82%) out of 60 isolates of ESBL-producing *E. coli* were confirmed to be *bla*_{CTX-M} type and were negative for *bla*_{TEM} and *bla*_{SHV} genes. This is the first study to demonstrate the occurrence of ESBL-producing *E. coli* in imported raw frozen mackerel fish in Saudi Arabia and the study result indicates that the mackerel fish might be the possible reservoir of *bla*_{CTX-M} gene and may contribute to the dissemination and transfer of these β -lactamase genes to humans through food chain. The high rate of occurrence of ESBL-producing *E. coli* in the mackerel fish indicates that there is an established reservoir of these bacteria in the mackerel fish. Further national wide studies are necessary to assess future trends in imported fish to Saudi Arabia.

Key words: Mackerel fish, *Escherichia coli*, extended-spectrum β -lactamase (ESBL), antimicrobial resistance, PCR.

INTRODUCTION

The increase and spread of extended spectrum beta-lactamase (ESBL) producing Enterobacteriaceae over

the past decade has become a global problem (Bradford, 2001; Paterson and Bonomo, 2005; Babic et al., 2006;

Bush, 2008; Ozcahar et al., 2011). The ESBLs are plasmid-encoded enzymes that inactivate a large number of β -lactam antibiotics such as extended-spectrum, broad-spectrum cephalosporins and monobactams. These β -lactamases are commonly inhibited by β -lactamase inhibitors, such as clavulanic acid, sulbactam, and tazobactam (Bush et al., 1995; Paterson and Bonomo, 2005). In several studies, across the globe reported alarming high rate of ESBL producing *Escherichia coli* not only in human infections (Paterson and Bonomo, 2005; Perez et al., 2007; Cantón et al., 2008; Poeta et al., 2008), but also in a wide range of food producing animals, (Ojer-Usoz et al., 2013), food products (Schmid et al., 2013) and environment (Mesa et al., 2006), showing that ESBL are not restricted to clinical settings alone. The microbiological safety of fish and other fishery products is an important public health concern throughout the world (FAO, 2010). A recent study from China has identified fish as a reservoir of ESBL producing *E. coli* (Jiang et al., 2012). Since the ESBLs are located on plasmids, many of them are derived from mutations in *bla*_{SHV} (Sulphydryl variable) and *bla*_{TEM} (Temoneira) genes determined by amino acid substitutions around the active site. Apart from SHV and TEM types, *E. coli* isolates may additionally produce CTX-M (cefotaximase-Munchen) enzymes. CTX-M β -lactamases are more active against cefotaxime and ceftriaxone than against ceftazidime, even though point mutations can increase their activity against ceftazidime as well (Manoharan et al., 2011). The CTX-M enzymes are being discovered throughout the world and are becoming the most prevalent beta-lactamases found in clinical isolates and now is considered the most prevalent ESBLs worldwide (Federico et al., 2007; Livermore et al., 2007; Bonnet, 2004; Canton and Coque, 2006). The CTX-M enzymes have been reported to be detected from different food products and food producing animals that were recognized as reservoirs for ESBL-producing *E. coli* (Carattoli, 2008; Geser et al., 2011; Egea et al., 2012). Recently, several studies have shown that these resistance genes entered and disseminated through the food chain via direct contact with humans and animals and could contribute to the spread of the these strains (Winokur et al., 2001; Oppegaard et al., 2001; Mesa et al., 2006; Egea et al., 2012).

There is no data available about ESBL-producing bacteria in food and aquaculture products and food of animal's origin in Saudi Arabia. Therefore, this study was conducted to estimate the incidence and to provide current baseline information on the antimicrobial resistance patterns and molecular characterization of ESBL-producing

E. coli in imported mackerel fish purchased from several retails supermarkets in the Eastern Province of Saudi Arabia.

MATERIALS AND METHODS

Sampling and isolation of ESBL producing *E. coli*

A total of 45 imported mackerel fish samples with labeled information (the country of origin and the storage temperature) were purchased from different supermarkets in Eastern Province of Saudi Arabia. The purchased samples were collected and transported on ice bag to the Microbiology Research Laboratory, University of Dammam. Upon arrival, the samples were kept intact on ice and analyzed within 2 to 3 h of collection. The examination of the mackerel fish samples were carried out according to the Methods of Bacteriological Analytical Manual (BAM, 2011) and also other published protocol with modifications (Elhadi et al., 2004; Zhao et al., 2001). Briefly, 25 g of samples (fish gills, intestines parts and skin) were placed into a stomacher bag containing 225 ml of EC broth (Oxoid, UK) and homogenized using a stomacher (Seward Stomacher 400 Circulator, UK) for 2 min and incubated for 18 to 24 h at 35°C. After enrichment incubation, 0.1 ml was streaked on ESBL chromogenic agar and incubated overnight at 37°C, three to four pink to reddish colored colonies with distinct morphological were isolated and subjected to biochemical tests (indole positive and oxidase negative) and were further confirmed by using API 20E (bioMe'rieux, France).

Antibiotic susceptibility testing

Antimicrobial susceptibility was determined by the disk diffusion method on Muller-Hinton agar Plates (Oxoid, Baringstoke, Hampshire, United Kingdom) as recommended by the Clinical and Laboratory Standards Institute (CLSI, 2010). The isolates were tested against the following antibiotics (Baringstoke, Hampshire, United Kingdom) AK: Amikacin (30 μ g); AP: Ampicillin (10 μ g); AUG: augmentin (30 μ g); ATM: aztreonam (30 μ g); FEP: cefepime (30 μ g); CTX: cefotaxime (30 μ g); CAZ: ceftazidime (30 μ g); CRO: ceftriaxone (30 μ g); C: chloramphenicol (30 μ g); CIP: ciprofloxacin (5 μ g); ETP: ertapenem (10 μ g); GM: gentamicin (10 μ g); K: kanamycin (30 μ g); NA: nalidixic acid (30 μ g); NI: nitrofurantoin (300 μ g); NOR: noroxin (10 μ g); PRL: piperacillin (100 μ g); T: tetracycline (30 μ g); TN: tobramycin (10 μ g); TM: trimethoprim (5 μ g) and TS: Trimethoprim/sulfamethoxazole (25 μ g). *E. coli* American Type Culture Collection (ATCC) 25922 was used as a reference strain for antimicrobial disk control.

Phenotypic confirmation of ESBL by E-test

E-test strips (Oxoid, UK) with concentration gradient of cefotaxime or ceftazidime at one end and cefotaxime or ceftazidime with Clavanic acid at the other end were accomplished according to the guidelines of the manufacturer for confirming ESBL production. ESBL production was determined by a ≥ 3 doubling dilutions decrease in the MIC of cefotaxime or ceftazidime in the presence of Clavanic acid. ESBL production was also recognized by the

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Abbreviations: ESBL, Extended-spectrum β -lactamase; ND, non-determinable; BAM, bacteriological analytical manual.

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Table 1. Nucleotide sequences of PCR primers.

Gene	Primer sequence (5'-3')	Amplicon size (bp)
<i>bla</i> _{CTX-M}	F: CGCTGTTGTTAGGAAGTGTG	754
	R: GGCTGGGTGAAGTAAGTGAC	
<i>bla</i> _{TEM}	F: TTTCGTGTCGCCCTTATTCC	403
	R: CGTTGTCAGAAGTAAGTTGG	
<i>bla</i> _{SHV}	F: CGCCTGTGTATTATCTCCCT	293
	R: CGAGTAGTCCACCAGATCCT	

Table 2. Percentage of antibiotic agents tested against *E. coli* strains isolated from imported mackerel fish (n=60).

Antimicrobial agents (μ g)	Resistant, n (%)	Intermediate, n (%)	Susceptible, n (%)
Amikacin (30)	0	0	60 (100)
Ampicillin (10)	60 (100)	0	0
Augmentin (30)	1 (1.7)	0	59 (98.3)
Aztreonam (30)	0	50 (83.3)	10 (16.7)
Cefepime (30)	0	0	60 (100)
Cefotaxime (30)	56 (93.3)	4 (6.7)	0
Ceftazidime (30)	0	0	60 (100)
Ceftriaxone (30)	56 (93.3)	4 (6.7)	0
Chloramphenicol (30)	9 (15)	0	41 (68.3)
Ciprofloxacin (5)	9 (15)	0	51 (85)
Ertapenem (10)	0	0	60 (100)
Gentamicin (10)	0	0	60 (100)
Kanamycin (30)	0	0	60 (100)
Nalidixic acid (30)	24 (40)	0	36 (60)
Nitrofurantoin (30)	3 (5)	0	57 (95)
Noroxin (10)	7 (11.7)	0	53 (88.3)
Piperacillin (10)	58 (96.7)	0	2 (3.3)
Tetracycline (30)	56 (53.3)	0	4 (6.7)
Tobramycin (10)	2 (3.3)	0	58 (96.7)
Trimethoprim (5)	18 (30)	0	42 (70)
Trimethoprim/sulfamethoxazole (25)	18 (30)	0	42 (70)

appearance of a phantom zone in the cefotaxime or ceftazidime strip. A non-determinable (ND) result was declared when the MICs were greater than the range of MICs of the respective E-test ESBL test strip.

Genotypic characterization of β -lactamases

All the isolates confirmed as ESBL producers were analyzed by using PCR amplification (Pitout et al., 1998; Poeta et al., 2008; Woodford et al., 2005). Genomic DNA was extracted by a standard heat lysis protocol and the same were used as the template. Amplification of TEM, SHV and CTX-M was performed with primer and cycling conditions presented in Table 1.

RESULTS AND DISCUSSION

Isolation of ESBL producing *E. coli*

A total of 60 isolates of ESBL-producing *E. coli* were

retrieved from 45 frozen imported mackerel fish samples that were obtained from supermarkets in Eastern Province of Saudi Arabia. In the present study, ChromID ESBL agar plates were used according to manufacturer's guidelines. It was found to have excellent sensitivity and specificity in detecting ESBL producing *E. coli* resulting in reduction in time and amount of bench work.

Antibiotic susceptibility testing

Antibiotic susceptibility testing result are shown in Table 2. Among all the total 60 study isolates, the higher percentage of resistance were found with piperacillin (96.7%), cefotaxime (93.3%) and ceftriaxone (93.3%) while, lowest resistance found with Tobramycin (3.3%) and Nitrofurantoin (5%). All the isolates were found to be

Table 3. Antibiotic resistant patterns and number of ESBL producing gene of *E. coli* strains isolated from imported mackerel fish (n=60).

Antibiotic resistance pattern	Number of isolates	ESBL genes		
		<i>bla</i> _{CTX-M}	<i>bla</i> _{TEM}	<i>bla</i> _{SHV}
		n (%)	n (%)	n (%)
AP-PRL	1	1	0	0
AP-NI-AUG	1	0	0	0
AP-CRO-PRL	2	2	0	0
AP-T-CRO-CTX-PRL	23	20	0	0
AP-T-CRO-CTX-PRL-NA	15	13	0	0
AP-T-CRO-CTX-PRL-TS-TM	9	5	0	0
AP-T-C-NI-CTX-PRL-NA-TS-CIP-TN-TM	2	2	0	0
AP-T-C-CRO-CTX-PRL-NA-TS-CIP-NOR-TM	7	6	0	0
Total	60	49 (81.7)	0	0

resistant to ampicillin whereas, all isolates were found to be susceptible to amikacin, cefepime, ceftazidime, ertapenem, gentamicin, and kanamycin. Unexpectedly, one isolate was found resistant against augmentin. 83.3% isolates had intermediate susceptibility against Aztreonam raising serious concern. This decreasing Aztreonam susceptibility was in concordance to our recent unpublished study regarding antibiotic resistant *E. coli* in beef and shrimp isolated from the same region. Out of all the 60 strains of *E. coli*, 23 strains yielded the similar resistance pattern AP-T-CRO-CTX-PRL, 15 strains yielded resistance pattern AP-T-CRO-CTX-PRL-NA and 9 strains yielded resistance pattern AP-T-CRO-CTX-PRL-TS-TM, respectively (Table 3). All the study isolates were found to be resistant to two or more antibiotics. The least discovered resistance patterns were AP-PRL and AP-NI-AUG, found in only one isolate each. A wide range of antibiotic classes are being used extensively in aquaculture, including aminopenicillins, amphenicols, macrolides, aminoglycosides, nitrofurans, fluoroquinolones, sulphonamides and tetracyclines (Heuer et al., 2009). The main consequence of this is the selection of MDR strains in the gut flora of fishes. During consumption of such contaminated fish products, the MDR strains are transferred to human gut and enter the food cycle (Heuer et al., 2009).

A recent research data from Sweden has shown the acquisition of fecal carriage of CTX-M-type ESBL producing *E. coli* in travelers to different parts of the world from Sweden and with 32% acquisition rate after travelling to Asia (Tangden et al., 2010). Fecal carriage is believed to be the most important reservoir of ESBL-producing bacteria in the community (Kluytmans et al., 2013). Some published research data documented the contamination of food animals, retailed chicken meat and beef meat with the ESBL-producing *Enterobacteriaceae* (Smet et al., 2008; Machado et al., 2008; Doi et al., 2010; Overdeest et al., 2011; Kluytmans et al., 2013). Previous reports suggest that resistant *E. coli* strains are

probably more likely to be transmitted from poultry to humans than are susceptible variants (Johnson et al., 2007). A recent survey comparing resistance rates among *E. coli* from humans vs. from poultry, pigs, and cattle in 11 European countries found strong, statistically significant correlations for various groups of antibiotics (Vieira et al., 2011).

Notably, for resistance to extended-spectrum cephalosporins, a significant correlation was found only between humans and poultry, implicating poultry as an important source for human-associated ESBL-producing *E. coli*. Infections with the antibiotic resistant bacteria and ESBL producing *Enterobacteriaceae* are associated with increased morbidity, mortality, and healthcare costs as a result of hospital acquired infections (Cosgrove, 2006; Tumbarello et al., 2010). ESBL-producing *Enterobacteriaceae* infections are increasingly frequent among community-dwelling patients without a history of hospitalization or antimicrobial use (Friedmann et al., 2009; Valverde et al., 2004; Dubois et al., 2010). Based on this finding imported mackerel fish is an important reservoir for ESBL-producing *E. coli*. Consumption of this contaminated fish with ESBL producing *E. coli* may lead to the transmission of genetic elements containing resistance genes to the human intestinal micro-biota. The abundant use of antimicrobial agents in a production animal poses threat to human health. This has recently spurred the US Food and Drug Administration to propose a ban on certain uses of cephalosporins for livestock (Schmidt, 2012).

Genotypic characterization of β -lactamases

All the 60 ESBL producing *E. coli* isolates were subjected to PCR analysis and 49 (82%) were positive for CTX-M and negative for TEM and SHV genes as shown in Table 3, Figures 1 and 2. The one isolate which was found resistant to Aztreonam was negative for CTX-M. The CTX-M production was highest among the *E. coli*

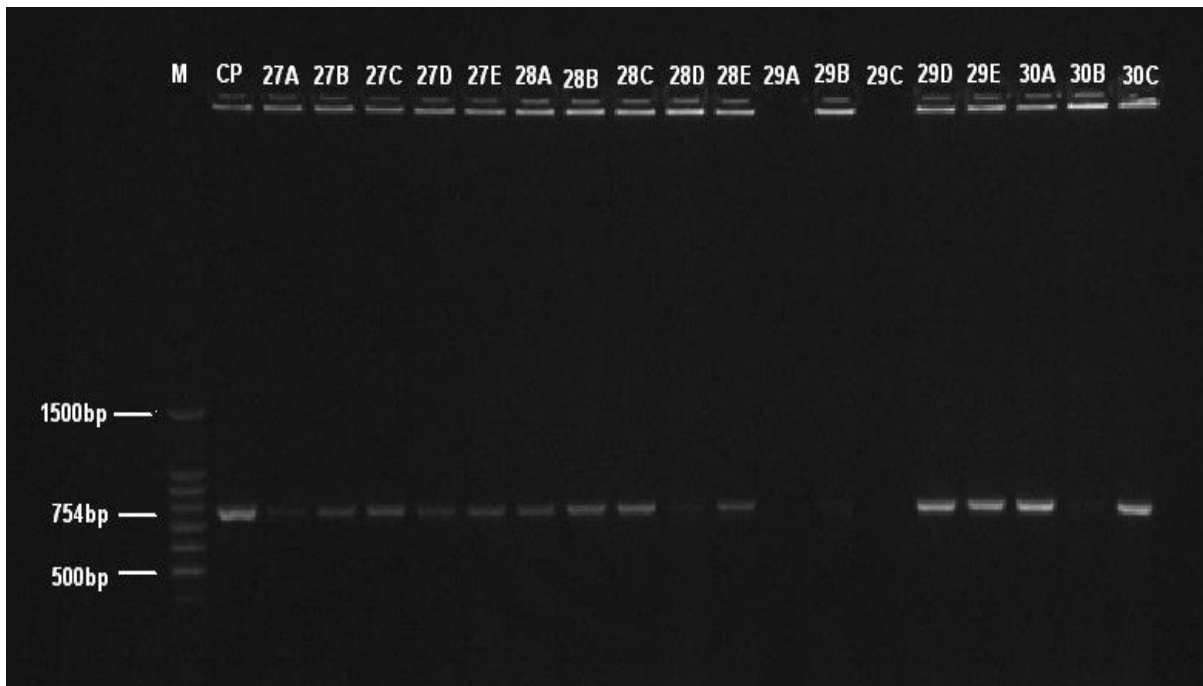


Figure 1. Representative Agarose gel electrophoresis of DNA amplification of selected *E. coli* isolates obtained with the PCR method. Lane M: Bench Top 100 bp DNA ladder (Promega, USA); Lane CP: Positive control. Lane 27A, 27B, 27C, 27D, 27E, 28A, 28B, 28C, 28E, 29D, 29E, 30A, 30C, CTX-M positive isolates. Lane 28D, 29A, 29B, 29C, and 30B, CTX-M negative isolates.

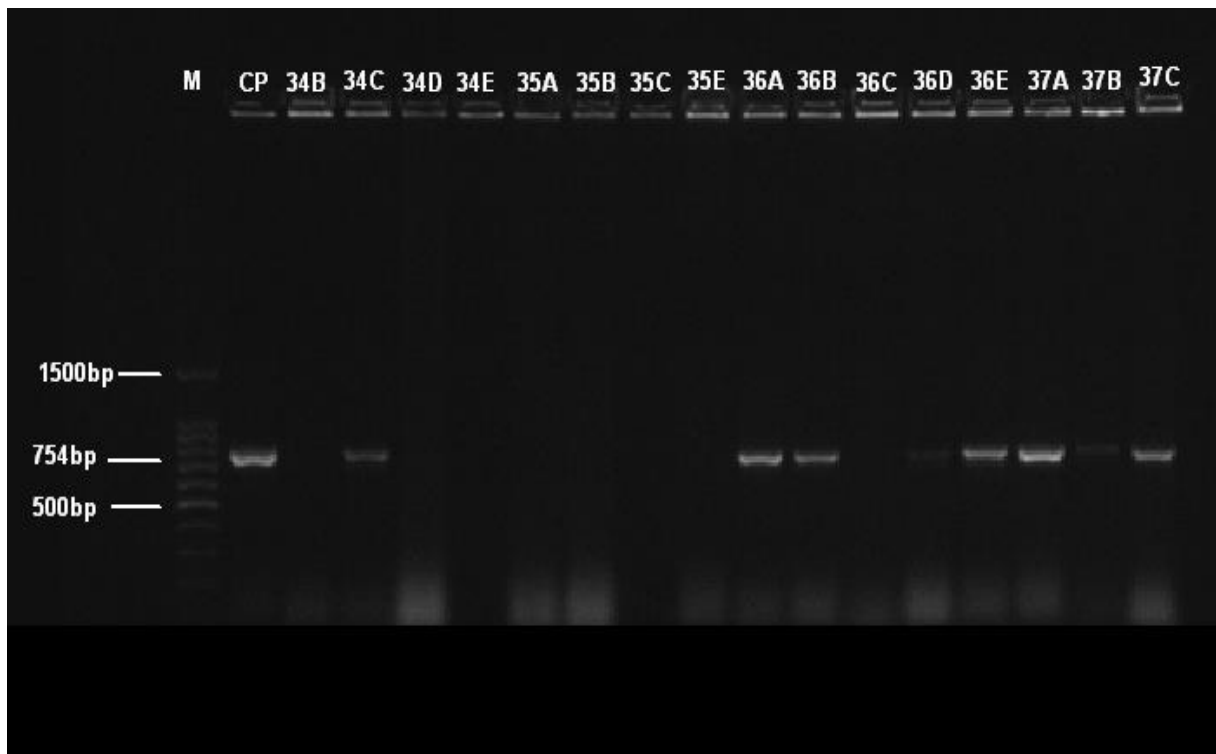


Figure 2. Representative Agarose gel electrophoresis of DNA amplification of selected *E. coli* isolates obtained with the PCR method. Lane M: Bench Top 100 bp DNA ladder (Promega, USA); Lane CP, Positive control. Lane 34C, 36A, 36B, 36E, 37A, and 37C: CTX-M positive isolates. Lane 34B, 34D, 34E, 35A, 35B, 35C, 35E, 36C, 36D, 37B: CTX-M negative isolates.

presenting the resistance pattern AP-T-CRO-CTX-PRL. TEM and SHV genes are often reported in ESBL-producing *Enterobacteriaceae* isolated from Poultry meat (Machado et al., 2008; Kola et al., 2012). The ESBL producing *E. coli* with CTX-M type and negative *bla*_{SHV} have been reported in wild birds in Germany (Guenther et al., 2010). Recent study from China demonstrated the presence of ESBL genes in farmed fish produced (Jiang et al., 2012). Infections due to ESBL-producing *E. coli* harboring ESBLs of the CTX-M classes have dramatically increased among human populations, particularly in the community setting (Livermore et al., 2007). There is very limited data available on the occurrence or prevalence of ESBL-producing *Enterobacteriaceae* in marine fish and in imported fish to Saudi Arabia. There is only one study to best of our knowledge, reported from China by Jiang et al. (2012) that described the prevalence of β -lactamase in *E. coli* isolated from farmed fish (Jiang et al., 2012). There is no data available on "pubmed" search (<http://www.ncbi.nlm.nih.gov/>) for Saudi Arabia and other Middle Eastern countries. As seen in our study, a predominance of CTX-M gene in *E. coli* isolated from food producing animals was reported in some European countries (Aarestrup et al., 2006; Girlich et al., 2007; Gonçalves et al., 2010).

Conclusion

The result of this study raises serious food safety concerns regarding the high prevalence of ESBL producing *E. coli* in the mackerel fish. This is the first study to report the high prevalence of ESBL-producing *E. coli* in the imported mackerel fish in Eastern Province of Saudi Arabia. The extensive prevalence of extended spectrum β -lactamase producing genes and high levels of co-resistance in *E. coli* detected in this study are of great concern and efforts should be made to monitor antibiotic resistance in aquaculture products, as this represents a major reservoir of antibiotic resistance. Larger studies should be undertaken in different geographical regions of the country to address these issues.

Conflict of interests

The author(s) did not declare any conflict of interest.

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