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Molecular detection of some antibiotic resistance genes of *Escherichia coli* isolated from bovine subclinical mastitis

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ABSTRACT

Antimicrobial drug resistance is considered an urgent major global public health threat facing humanity. With the rise in the prevalence and severity of both fatal and crippling illnesses, this crisis will have a catastrophic effect on human society. It doesn't only affect public health but also causes serious problems in the dairy industry. The objectives of this study were to determine the prevalence, antibiotic resistance, and detection of *Escherichia coli* that produces extended-spectrum β-lactamase (ESBL) isolated from bovine subclinical mastitis cases. *Escherichia coli* was detected in 26 out of the 100 subclinical mastitis cases. The antibiotic sensitivity revealed that 10 from 26 isolated *Escherichia coli* were multidrug resistant. The isolates were most frequently resistant to amoxicillin (AMX) at 53.85%, ampicillin (AMP) at 46.1%, cefotaxime (CTX) at 42.3% followed by amoxicillin-clavulanic acid (AMC) at 38.5%. All the 26 *Escherichia coli* isolates were tested for Extended Spectrum b-lactamase by using the disc diffusion method, and the same 10 multidrug- resistant isolates were positive for Extended Spectrum b-Lactamases. All ten multidrug resistance and Extended Spectrum b-Lactamases *Escherichia coli* isolates were identified genetically by the *Pho*A gene and were found harboured b-Lactamases antibiotic resistance genes bla_{TEM} 100%, bla_{CTXM} 90%, bla_{SHV} 80%, and *amp*C 80% respectively. The obtained results showed that phenotypic detection of 10 multidrug resistance and Extended Spectrum b-Lactamases isolates were agreed with genotypic molecular detection of b-Lactamases antibiotic resistance genes.

Introduction

Worldwide, bovine mastitis is one of the most serious and costly diseases in the dairy business. 137 distinct bacteria have been linked to mastitis in cows, although *Escherichia coli* is one of the most frequent culprits (Kempf *et al*., 2016; Yang *et al*., 2016).

Subclinical mastitis is a common global health issue that causes significant alterations in the composition of milk and several unfavorable effects in dairy farms. Due to its influence on the quantity and quality of milk produced, subclinical mastitis is overemphasized from an economic standpoint (Ruegg and Reinemann, 2002).

Moreover, subclinical mastitis raised the possibility of antimicrobial residuals in milk and aided in culling (McFadden, 2011). Among the greatest prevalent bacteria, *Escherichia coli* has been connected to both antibiotic resistance and subclinical mastitis (Hinthong *et al*., 2017). Bad hygienic practices in the animal environment are typically linked to *E. coli* mastitis (Abdel-Tawab *et al*., 2018).

One of the formidable challenges to humanity is antibiotic resistance. Antimicrobial resistance is expected to be the cause of ten million annual deaths by 2050, according to numerous recent research (Sugden *et al*., 2016). The overuse of these agents by humans and animals leads to the emergence of multidrug-resistant *E. coli*, which consequently threatens their lives. Multidrug-resistant *E. coli* was isolated from food samples including raw milk in Egypt (Aly *et al*., 2012), and milk products (Samy *et al*., 2022).

Sadly, *E. coli* bacteria are becoming increasingly resistant to the majority of beta-lactam antibiotic. One of the most dramatic ways that antibiotic resistance against β-lactam drugs can arise is by the synthesis of β-lactamase enzyme. The antibiotic's β-lactam ring is hydrolyzed by the β-lactamase enzyme, making it inactive (Geser *et al*., 2012). Enzymes that are inhibited by clavulanic acid and effective against a wide range of β-lactams are known as ESBLs. They are grouped into major families: TEM, SHV, CTX-M and OXA (Bradford, 2001).

Therefore, The current study was done to add recent data to the prevalence and antibiotic susceptibility profiles of *E. coli* strains isolated from bovine subclinical mastitis and to determine the prevalence of ESBL-producing *E. coli* as an important mastitic pathogen and some of its most important β-lactam antibiotic resistance genes.

Materials and methods

Ethical approval

The experiments were performed on naturally collected milk samples, no ethical question was raised by this study. The samples were taken under the supervision of a veterinarian.

Sample collection

Aseptic milk samples (n = 188) were taken from dairy cows that were nursing at small-holder farms in Assiut governorate, Egypt. Following the removal of the initial streams, the milk samples were collected in sterile falcon tubes and brought to the laboratory within two hours in an ice box kept at 4°C. The California Mastitis Test (CMT) was performed on the samples (Ghanbarpour and Oswald, 2010; Ameen *et al*., 2019).

California mastitis test

By indirectly estimating the Somatic Cell Count (SCC) in milk, the California Mastitis Test (CMT) was used as a screening test for subclinical mastitis (Leach *et al*., 2008). The test reagent (COVETO, Montaigu, France) and an equivalent volume of milk were put to a four-well plastic paddle,

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which was then carefully and gently manually agitated. The result was interpreted as negative, trace, 1+, 2+, and 3+, as described by Schalm *et al*. (1971). All the positive CMT milk samples (n=100) were tested microbiologically for isolation and identification of *E. coli*.

Isolation and identification of E. coli isolates

The CMT positive milk samples (n=100) were cultivated into MacConkey broth and incubated aerobically at 37°C for 24 h, then a loopful from the broth was cultured on MacConkey (MAC) agar (Merck, Germany) and incubated aerobically at 37°C for 24 h. The suspected *E. coli* pink colonies were streaked on Eosin Methylene Blue Agar (EMB), and incubated for 24 h at 37°C (Leininger *et al*., 2001; Soomro *et al*., 2002; Disassa *et al*., 2017). Colonies with m*et al*lic sheen were picked up and subcultured on nutrient agar slope for morphological and biochemical examination, according to Quinn *et al*. (2011).

Antibiotic sensitivity testing

Antibiotic susceptibility testing was performed by Kirby Bauer disc diffusion method on Mueller Hinton agar plate media according to the Clinical Laboratory Standards Institute guidelines (Sigma-Aldrich, USA) for the isolated *E. coli* strains against 8 antimicrobial agents, and the results were interpreted according to CLSI (2020). The used antimicrobials agents were ampicillin (AMP, 10 µg), amoxicillin (AMX 20 µg), amoxicillin-clavulanic acid (AMC, 20/10 µg), cefotaxime (CTX, 30 µg), colistin (CL, 10 µg), gentamicin (GEN, 10 µg), tetracycline (TE, 30 µg) and trimethoprim-sulfamethoxazole (COT, 1.25/23.75 µg). The diameter of the inhibition zone produced by each antibiotic disc was measured and interpreted using the CLSI zone diameter interpretative standards (CLSI, 2020). Isolates resistant to three or more antimicrobial categories were classified as multidrug resistant (MDR) (Magiorakos *et al*., 2012).

Extended Spectrum B-Lactamases (ESBL) testing

ESBL producing *E. coli* isolates were identified by using the double-disc synergy test (DDST) diffusion method to detect ESBL production. Cefotaxime (CTX 30 µg), and amoxicillin-clavulanic acid (AMC 20/10 µg) were used for testing. A zone diameter of more than 5 mm between cefotaxime and cefotaxime-clavulanic acid was considered ESBL positive (CLSI, 2020).

Detection of antibiotic resistance genes

All the ten Phenotypic ESBL producing and multidrug-resistant *E. coli* isolates were molecular identified by *Pho*A gene and screened by polymerase chain reaction (PCR) for detection of some β-lactam antibiotic resistance genes (bla_{TEM}, bla_{CTXM}, bla_{SHV}, and *amp*C) using specific primers that were supplied from Metabion (Germany) as shown in (Table 1). DNA was extracted using QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations, PCR amplification and analysis of the PCR Products was completed as mentioned previously by Sadek and Koriem (2022). Application of PCR assay was performed in Reference Lab for veterinary quality control on poultry production, Animal Health Research Institute, Doki, Giza, Egypt.

Results

The California Mastitis Test revealed that 100 (53.2%) out of the 188 milk samples that were collected and tested for subclinical mastitis were positive for California Mastitis Testing (CMT). Based on a bacteriological examination, 26 (26%) out of 100 subclinical mastitic milk samples had evidence for *E. coli* isolation and identification. Ten of the 26 *E. coli* isolates that were studied showed multidrug resistance and were positive for extended-spectrum b-lactamase (ESBL) testing. The results of

Table 1. The primer sequences, target genes, and PCR settings were utilized in the genomic study of *E. coli* isolates.

Target gene	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
bla_{TEM}	ATCAGCAATAAACCAGC	516	94˚C	94˚C	54˚C	72˚C	72˚C	Colom *et al*. (2003)
	CCCCGAAGAACGTTTTC		5 min.	30 sec.	40 sec.	45 sec.	10 min.	
bla_{SHV}	AGGATTGACTGCCTTTTTG	392	94˚C	94˚C	54˚C	72˚C	72˚C	
	ATTTGCTGATTTCGCTCG		5 min.	30 sec.	40 sec.	40 sec.	10 min.	
Bla_{CTX-M}	ATG TGC AGY ACC AGT AAR GTK ATG GC	593	94˚C	94˚C	54˚C	72˚C	72˚C	Archambault *et al.* (2006)
	TGG GTR AAR TAR GTS ACC AGA AYC AGC GG		5 min.	30 sec.	40 sec.	45 sec.	10 min.	
ampC	TTCTATCAAMACTGGCARCC	550	94˚C	94˚C	50˚C	72˚C	72˚C	Srinivasan *et al.* (2005)
	CCYTTTTATGTACCCAYGA		5 min.	30 sec.	40 sec.	45 sec.	10 min.	
phoA	CGATTCTGGAAATGGCAAAAG	720	94˚C	94˚C	55˚C	72˚C	72˚C	Hu *et al.* (2011)
	CGTGATCAGCGGTGACTATGAC		5 min.	30 sec.	40 sec.	45 sec.	10 min.	

Table 2. Antibiotic sensitivity results for *E. coli* isolated from subclinical mastitic milk (n =26).

Antibiotics	Sensitive		Intermediate		Resistant	
	No	%	No	%	No	%
ampicillin (AMP)	6	23.1	8	30.8	12	46.1
amoxicillin (AMX)	1	3.85	11	42.3	14	53.85
amoxicillin-clavulanic acid (AMC)	15	57.7	1	3.8	10	38.5
tetracycline (TE)	17	65.4	3	11.5	6	23.1
cefotaxime (CTX)	14	53.85	1	3.85	11	42.3
gentamicin (GEN)	21	80.8	4	15.4	1	3.8
colistin (CL)	24	92.4	1	3.8	1	3.8
trimethoprim-sulfamethoxazole (COT)	22	84.7	1	3.8	3	11.5

PCR showing that the ten multidrug resistance and Extended Spectrum b-Lactamases *Escherichia coli* isolates were confirmed genetically by the *pho*A gene and were found harboured *B*-Lactamases antibiotic resistance genes bla_{TEM} 100%, bla_{CTXM} 90%, bla_{SHV} 80 %, and *amp*C 80 % (Figs.1,2,3,4 and 5). The obtained results showed that phenotypic detection of 10 multidrug resistance and Extended Spectrum *B*-Lactamases isolates were agreed with genotypic molecular detection of *B*-Lactamases antibiotic resistance genes.

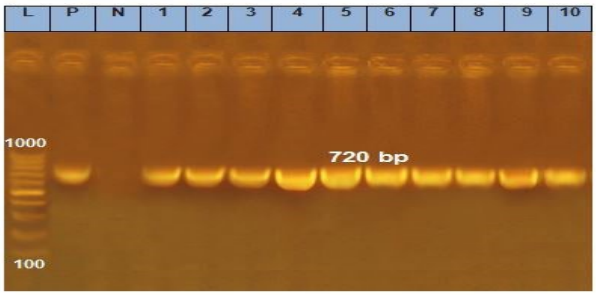

Fig. 1. patterns of agarose gel electrophoresis for the *Escherichia coli pho*A gene PCR amplification products. Lane L, DNA ladder marker (100 bp). Lane P, control positive *Escherichia coli pho*A gene (720 bp). Lane N, negative control. Lanes 1 to 10 positive *Escherichia coli* isolates for *pho*A gene.

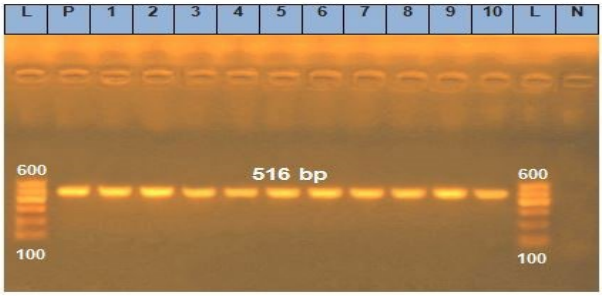

Fig. 2. patterns of Agarose gel electrophoresis for PCR amplification products of the bla_{TEM} gene in *Escherichia coli*. Lane L, DNA ladder marker (100 bp). Lane P, control positive *Escherichia coli* bla_{TEM} gene (516 bp). Lane N, negative control. Lanes 1 to 10 positive *Escherichia coli* isolates for bla_{TEM} gene.

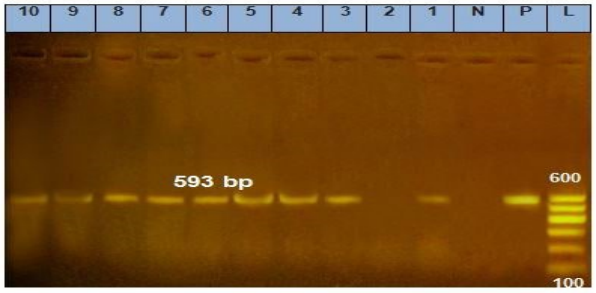

Fig. 3. patterns of agarose gel electrophoresis for PCR amplification products of the Bla_{CTX-M} gene in *Escherichia coli*. Lane L, DNA ladder marker (100 bp). Lane P, control positive *Escherichia coli* bla_{CTX-M} gene (593 bp). Lane N, negative control. Lanes 1 and 3 to 10 positive *Escherichia coli* isolates for Bla_{CTX-M} gene,lane 2 negative *Escherichia coli* isolates for bla_{CTX-M} gene.

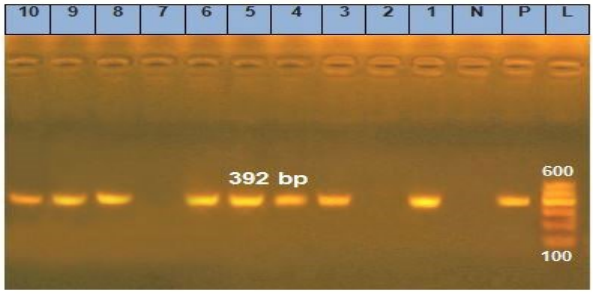

Fig. 4. patterns of agarose gel electrophoresis for PCR amplification products of the bla_{SHV} gene in *Escherichia coli*. Lane L, DNA ladder marker (100 bp). Lane P, control positive *Escherichia coli* bla_{SHV} gene (392 bp). Lane N, negative control. Lanes 1,3,4,5,6,8,9 and 10 positive *Escherichia coli* isolates for bla_{SHV} gene,Lane 2 and 7 negative *Escherichia coli* isolates for bla_{SHV} gene.

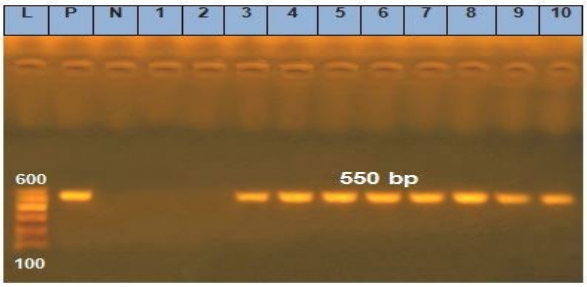

Fig. 5. Patterns of agarose gel electrophoresis for the *amp*C gene of *Escherichia coli* obtained from PCR amplification. Lane L, DNA ladder marker (100 bp). Lane P, control positive *Escherichia coli amp*C gene (550 bp). Lane N, negative control. Lanes 3 to 10 positive *Escherichia coli* isolates for *amp*C gene, lane 1 and 2 negative *Escherichia coli* isolates for *amp*C gene.

Discussion

Sub-clinical mastitis is one of the essential causes of economic losses in dairy farm specially with the extensive misuse of beta-lactam antibiotics for the treatment of this disease (Das *et al*., 2017). Over the last few years, there has been a growing global concern for veterinary and public health due to the isolation of ESBL-producing *E. coli* from food-producing animals (Seiffert *et al*., 2013). Consequently, the goal of the current investigation was to ascertain the prevalence of ESBL-producing *E. coli* as an important mastitic pathogen and to ascertain the antimicrobial resistance profile and some of the most significant β-lactam antibiotic resistance genes of ESBL-producing *E. coli*.

To minimize production losses and improve recovery chances, mastitis should be diagnosed as soon as possible. Additionally, for mastitis in dairy animals to be successfully controlled, it is imperative that the animals which are subclinically afflicted must be identified (Ahmed *et al*., 2008).

Based on the findings of California Mastitis Test (CMT), the study found that 53.2% of dairy cattle had subclinical mastitis. our findings were in a close agreement with the result reported by Gianneechini *et al*. (2002); Haltia *et al*. (2006); Rahman *et al*. (2010); Kabir *et al*. (2017) and Altaf *et al*. (2019) 52.4, 52.2, 53.1,51 and 57.68 respectively, while lower incidence recorded by Rafyi-Barzoki (1998); Zamani *et al*. (2004) and Hashemi *et al*. (2011) 33.2, 33.7 and 42.5 % respectively. Kivaria *et al*. (2004); Karimuribo *et al*. (2008); Argaw and Tolosa (2008); koriem (2014); Nesreen Bakr *et al*. (2019) and Ghallache *et al*. (2021) indicated that smaller holder farms had higher rates of subclinical mastitis in their nursing cows 90.3, 75.9, 89.54, 67.01, 84 and 66.4% respectively. The variations in results could be due to breed, parity, stage of lactation, and variances in farm management practices (Almaw *et al*., 2008).

In the current investigation, the incidence of isolated *E. coli* from the bovine subclinical mastitis milk samples (Positive CMT milk samples) was 26%. A lower incidence 9.3, 9.3, 9 and 9.1% were reported by Ombarak *et al*. (2019); Ahmed *et al*. (2021); El-Khabaz *et al*. (2022) and Subhi *et al*. (2023) respectively. Also, Lira *et al*. (2004); Momtaz (2010) and Abdel-Tawab *et al*. (2018) isolated *E. coli* from 8.5, 10.5 and 7.5% of the tested milk samples respectively. Nearly similar findings was reported by Momtaz *et al*. (2012) found that 57 of 181 mastitic milk samples tested were positive for *E. coli*, with percentage about 31.5% and Ameen *et al*. (2019) was 20%. Diverse countries and areas have shown variations in the frequency of *E. coli* isolation, which could be related to variations in climate, lactation season, nutrition, and diverse management circumstances (Marashifard *et al*., 2019).

The gained results (Table 2) of antimicrobial sensitivity testing showed that the isolates were most frequently resistance to amoxicillin (AMX), ampicillin (AMP), cefotaxime (CTX), amoxicillin-clavulanic acid (AMC) and tetracycline (TE) 53.85, 46.1,42.3,38.5 and 23.1 % respectively among *E. coli* isolates from bovine mastitis. These findings were nearly agreed with Ameen *et al*. (2019) and Subhi *et al*. (2023) they found that *Escherichia coli* that was isolated from milk samples exhibited multi-agent resistance. It displayed resistance to tetracycline, ampicillin, and amoxicillin-clavulanic acid. These outcomes were comparable to those of Skočková *et al*. (2015) which showed resistance of *E. coli* isolates to ampicillin, amoxicillin-clavulanic acid, tetracycline, and trimethoprim-sulfamethoxazole, and those of Samy *et al*. (2022) who detected resistance of *E. coli* isolates to oxytetracycline, amoxicillin, and ampicillin, and Ombarak *et al*. (2018) who found resistance to ampicillin, tetracycline, and trimethoprim-sulfamethoxazole among *E. coli* isolates. While Bag *et al*. (2021) recorded resistant *E. coli* isolates to amoxicillin-clavulanic acid (94.5%), followed by ampicillin (89.5%), and tetracycline (89.5%). The danger of multi-antibiotic resistance (MAR) *E. coli* is that it could transmit this drug resistance to humans as stated by Yoon and Lee (2022) besides causing its known infections and syndrome.

The obtained result revealed that the frequency of ESBL *E. coli* isolates from the Positive CMT milk samples in cattle were 38.46%, these results were higher than those of Filioussis *et al*. (2020) 6.7% and Subhi *et al*. (2023) 9%. while this result was higher than the findings of Dahmen *et al*. (2013) and Ali *et al*. (2017), whose results were 0.3 and 0.25% respectively. Similar findings were obtained by Shereen *et al*. (2022) 38.2 %. ESBLs are becoming more commonplace across the globe. This could be clarified and explained by the fact that the resistance genes are often carried on plasmids that are transposable, meaning they can be transferred between different strains of bacteria and between species, increasing their prevalence. Certain plasmid-mediated β-lactamases, like bla_{TEM}/$_{SHV}$, are mutant variants of certain ESBL genes, whereas others, like bla_{CTX-M}, are produced by environmental bacteria (Rupp and Fey 2003 and Overdevest *et al*., 2011).

According to the PCR results of *pho*A, bla_{TEM}, bla_{CTX-M}, bla_{SHV} and *amp*C genes were detected in the 10 *E. coli* isolates, which were found ESBL positive and multidrug resistance in percentages of 100, 100, 90, 80 and 80% respec¬tively (Figs. 1, 2, 3, 4 and 5). Penicillin, first and fourth generation cephalosporins, and monobactams are all ineffective against extended-spectrum beta-lactamases. The plasmid is typically linked to extended spectrum beta-lactamases. The beta-lactamase groups TEM, SHV, and CTX-M are the commonly seen in isolates of Enterobacteriaceae. The beta-lactamase genes TEM-1/TEM-2 and SHV-1 (bla_{TEM-1}/bla_{TEM-2} and bla_{SHV-1}) are the source of the TEM and SHV groups, conjugation may transmit the CTX-M gene (Pehlivanoğlu *et al*., 2017). According to the collected results, the most common ESBL genes were bla_{TEM} and bla_{CTX-M}, with bla_{SHV} and *amp*C coming in second and third. According to reports from China and other nations, CTX-M was shown to be the most common ESBL in *E. coli* from bovine mastitis. This finding agree with those reports by Freitag *et al*. (2016); Pehlivanoglu *et al*. (2016) and Feng *et al*. (2018). Furthermore, the obtained data support earlier research and show that CTX-M is the most prevalent kind of *E. coli* that causes bovine mastitis in China (Ali *et al*., 2016). *Escherichia coli* isolates showed the presence of the *amp*C gene (80%) that was nearly similar to the results of Ismail and Abutarbush (2020) in Jorden (86%) but was lower than those of Fazel *et al*. (2019) in Iran (92.8%) and Subhi *et al*. (2023) was (94.4 %). *Amp*C gene produces *Amp*C β-lactamase, which is the first known destroyer of the β-lactam ring of β-lactam antibiotics. This led to the incredible challenge of antibiotic resistance, which is today recognized as a serious public health issue and a growing global public health concern. Therefore, it is intriguing to investigate the phenotypic and genotypic characteristics of *Amp*C gene in relation to ABR (Bush and Bradford, 2016). Furthermore, *Amp*C β-lactamase has been demonstrated to have the ability to suppress a variety of antibacterial drugs.

Conclusion

Most of the *E. coli* isolates are MDR, with particular focus on ESBL. The study confirmed the prevalence and dissemination of the key antibiotic resistance genes (*bla*TEM, bla_{CTXM}, bla_{SHV}, and *amp*C), which are the most common ESBL genotypes, and discovered ESBL-producing *E. coli* in mastitic milk samples from bovine dairy farms. To stop the spread of these resistance genes in the future, which could have grave and disastrous health repercussions, preventive measures, and rigorous, ongoing surveillance of *E. coli* that produces ESBL are important. Authorities should follow the One Health concept to lessen the risk of new variations. The use of antibiotics on farms must be justified in order to stop the spread of resistant strains in animal and human populations. Antibiotics should never be used as growth enhancers.

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Conflict of interest

There is no conflict of interest declared by the authors.

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