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MOLECULAR CHARACTERIZATION OF ESBL AND COLISTIN RESISTANCE GENES AMONG CLINICAL ISOLATES OF GRAM-NEGATIVE BACTERIA IN A TERTIARY HOSPITAL IN ABAKALIKI, NIGERIA

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ABSTRACT: Antimicrobial resistance is a threat to public health. This study investigated the occurrence of ESBL and colistin resistance among *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* recovered from clinical samples (n=500) in a tertiary teaching hospital in Abakaliki, Nigeria. The samples were bacteriologically analyzed using selective culture media, biochemical tests, and PCR. All isolates were screened for ESBL and colistin resistance genes using both PCR and disk diffusion methods that included standard antibiotic disks for susceptibility studies. *E. coli* (n=173), *K. pneumoniae* (n=120) and *P. aeruginosa* (n=62) were recovered from the clinical samples investigated. The most prevalent ESBL genes detected is ^{bla}CTX-M found in 3 isolates of *P. aeruginosa*, 4 isolates of *E. coli* and 2 isolates of *K. pneumoniae*. The colistin resistance gene, *mcr-3* was detected in 4 strains of *E. coli* for the first time in Southeast Nigeria. Both *mcr-1* and *mcr-3* was not detected in the *K. pneumoniae* strains. Only the *mcr-2* colistin resistance genes were detected in *K. pneumoniae* strains. There was no detection of *mcr-2* gene in the *P. aeruginosa* strains. Over 50% of the ESBL and colistin-resistant isolates showed high levels of reduced susceptibility to the tested antibiotics. Imipenem, to which all the ESBL and colistin-resistant isolates showed complete susceptibility (100%), followed by gentamicin (85%), were the most effective antibiotic against the ESBL and colistin-resistant isolates. Surveillance of ESBL and colistin-resistant bacteria in hospitals is important to mitigate the evolution and transmission of these multidrug resistant pathogens within the hospital and general environment.

INTRODUCTION: Antimicrobial resistance (AMR) in pathogenic bacteria, particularly gram-negative bacteria is a growing public health threat that stretches the effectiveness of available antibacterial agents and makes it difficult for physicians to select the best antibiotic for therapy.

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Gram-negative bacterial pathogens harboursome mobile genetic elements that allow them to express multidrug resistance traits such as extended-spectrum beta-lactamases (ESBLs), metallo beta-lactamase and AmpC enzymes [1, 2, 3]. ESBLs are β-lactamases capable of conferring bacterial resistance to the third generation cephalosporins

but are inhibited by β-lactamase inhibitors such as clavulanic acid [2, 4, 5]. ESBL-producing bacteria are clinically relevant because they exhibit co-resistance to other antibiotics [6, 7]. The implication is that treatment options for treating infection caused by ESBL-producing bacteria become limited, leading to long hospitalization, infection persistence, and economic loss [8, 9]. With regards to the upsurge of multidrug-resistant infections, including those caused by ESBL-producing bacteria, colistin has re-established itself as a valuable therapeutic option, having been abandoned in the early 1980's because of its nephrotoxic and neurotoxic effects [5, 10].

Colistin (polymyxin E) whose mechanism of action is based on targeting the polyanionic lipid A of the lipopolysaccharides (LPS) in the outer membrane (OM) of Gram-negative bacteria, belongs to the polymyxin group of antibiotics which were originally isolated from the spore forming soil organism *Paenibacillus polymyxa* [11]. Though they have a narrow spectrum of activity, reports have shown that colistin is effective against members of the *Enterobacteriaceae* family [10, 12]. However, there have been several reports of the resistance of some pathogenic bacteria to the antimicrobial onslaught of colistin [13, 14]. In south-south and some regions of Nigeria, the prevalence of colistin resistance genes (*mcr*$^{-1}$) in Gram-negative bacteria, including *Escherichia coli* has been documented [15]. Also in Lebanon, colistin resistance was reported in clinical isolates of *Klebsiella pneumonia* [16]. In China, a high prevalence of colistin resistance (*mcr-1*) genes and strains have been reported in animals, food animals, and humans [13, 17, 18].

It is documented that some ESBL-producing bacteria also possess the *mcr*$^{-1}$ genes that allow these organisms to resist the antimicrobial activity of colistin [19]. Since, colistin is still used as a last-line antibiotic for the treatment of microbial infections, including those caused by multidrug-resistant bacteria such as those expressing ESBLs, in clinical settings, it is of public health importance to monitor the emergence and transmission of colistin-resistance genes and strains in both human and animal population. Colistin is used in livestock globally [19]. This may contribute to the emergence and spread of colistin-resistance genes and strains in both human and animal populations globally. The growing resistance of bacterial pathogens, including those producing ESBL, to colistin is a public health risk that demands urgent attention to protect the efficacy of colistin – as an important last-line antibiotic in clinical medicine. To bridge the knowledge gap and contribute new information on the dearth of data on colistin resistance in Nigeria, this study investigated the prevalence of ESBL and colistin resistance genes in clinical Gram-negative bacteria isolates of *E. coli, K. pneumoniae* and *P. aeruginosa*.

MATERIALS AND METHODS:

Ethical Approval and Sample Processing: The experimental protocols carried out in this study were approved by the Ethics and Research Committee of the State Ministry of Health, Ebonyi State, Nigeria (2017/65/C7002). A total of 500 non-duplicate clinical samples, including sputum (n=100), urine (n=100), wound (n=100), high vaginal swab (n=100), and feces(n=100), were collected and bacteriologically analyzed using selective culture media, biochemical testing and microscopy for the isolation of Gram negative bacteria including *E. coli, K. pneumoniae* and *P. aeruginosa*. The isolated bacteria were further characterized using 16 sRNA PCR techniques.

Test for Colistin Resistance and ESBL-Producing Bacteria: Turbid suspension of bacteria (0.5 McFarland turbidity standards) was used for experimenting. Colistin resistance was detected using E-test strips for measuring MIC (Minimum Inhibitory Concentration), while ESBL bacteria were phenotypically confirmed using double disk synergy test [2]. Bacterial isolates showing reduced susceptibility to any of the cephalosporins, including cefotaxime (CTX, 30µg) and ceftazidime (CAZ, 30µg) [Oxoid, UK], according to the Clinical Laboratory Standard Institute (CLSI) criteria were further confirmed for ESBL production using the double disk synergy test (DDST) method. For DDST, amoxicillin-clavulanic acid, AMC (20/10µg) was placed at the center of the Mueller-Hinton (MH) agar plate, and CTX (30µg) and CAZ (30µg) disk were each placed at a distance of 15mm from the central disc (AMC). The plates were incubated at 37^{o}C for 18-24 h. ESBL production was inferred phenotypically when the zones of inhibition of CAZ or CTX were expanded by AMC [2, 4].

Antibiotics Susceptibility Studies for Colistin-resistant and ESBL Bacteria: This was conducted using single antibiotic disks of colistin (10µg), cefepime (30µg), imipenem (10µg), amoxicillin-clavulanic acid (20/10µg), ceftazidime (30µg), ciprofloxacin, (5µg), ertapenem (10µg), aztreonam (10µg), nitrofurantoin (30µg), gentamicin (10µg), imipenem (10µg), ertapenem (10µg), and ofloxacin (5µg) [Oxoid, UK]on MH agar plate swabbed with bacterial suspension (0.5 McFarland turbidity standards). Susceptibility plates were incubated at 37^{o}C for 24h; and isolates were inferred as susceptible or resistant using the antibiotic breakpoints of CLSI [2, 20].

PCR Screening for ESBL Genes: Bacteria showing less susceptibility to any third-generation cephalosporins (e.g., CAZ, CTX) according to the CLSI protocol were phenotypically defined as ESBL bacteria [2, 4, 20]. ESBL bacteria were screened for ESBL genes (*^{bla}TEM*, *^{bla}SHV*, *^{bla}CTX-M*, *^{bla}*Oxa) using conventional PCR. DNA was extracted, purified, and quantified using the ZYMO DNA miniprep kit (Zymo Research Corporation, USA) and NanoDrop technique, respectively (Thermo Fischer Scientific, USA).

ESBL gene amplification was done using specific oligonucleotide primers **Table 1** in a thermal cycler (Lumex instruments, Canada). Amplified DNA products were run on a 1.5% agarose gel and visualized in a UV transilluminator (Scientico, India).

TABLE 1: PRIMERS FOR GENE AMPLIFICATION OF ESBL GENES

Gene target	Sequence	Amplicon size	Reference
^{bla}TEM	F-5'-ATTCTTGAAGACGAAAGGGC-3'	1150	[21]
	R-5'-ACGCTCAGTGGAACGAAAAC-3'		
^{bla}SHV	F-5'-CACTCAAGGATGTATTGTG-3'	885	[22]
	R-5'-TTAGCGTTGCCAGTGCTCG-3'		
^{bla}CTX-M	F-5'-GTTACAATGTGTGAGAAGCAG-3'	1041	[22]
	R-5'-CCGTTTCCGCTATTACAAAC-3'		
^{bla}Oxa	F-5'-ACACAATACATATCAACTTCGC-3'	813	[22]
	R-5'-AGTG TGTTTAGAATGGTGATC-3'		

PCR Screening for Colistin Resistance Gene: Colistin-resistant phenotypes were inferred according to the CLSI protocol [4, 20]. A conventional PCR technique was used to screen isolates for the presence of colistin resistance genes (*mcr$^{1-3}$*) [13, 17, 25]. DNA was extracted, purified and quantified using ZYMO DNA miniprep kit (Zymo Research Corporation, USA) and NanoDrop technique, respectively (Thermo Fischer Scientific, USA). The amplification of colistin resistance genes was done using specific primers **Table 2** in a thermal cycler (Lumex instruments, Canada). Amplified DNA products were run on a 1.5% agarose gel and visualized in a UV transilluminator (Scientico, India).

TABLE 2: PRIMERS FOR AMPLIFICATION OF COLISTIN RESISTANCE GENES

Gene target	Sequence	Amplicon size	Reference
mcr-1	F- 5′-CGGTCAGTCCGTTTG TTC -3′	309	[17]
	R- 5′-CTTGGTCGGTCTGTAGGG-3′		
mcr-2	F- 5-TGTTGCTTGTGCCGATTGGA -3′	567	[23]
	R-5-AGA TGGTATTGTTGGTTGCTG-3′		
mcr-3	F-5' -TTGGCACTGTATTTTGCATTT-3′	542	[24]
	R-5'-TTAACGAAATTGGCTGGAACA-3′		

RESULTS AND DISCUSSION: The percentage prevalence of Gram-negative bacteria isolated from the clinical samples bacteriologically analyzed in this study is shown in **Fig. 1**. *Escherichia coli* were the most prevalent bacteria isolated from the clinical samples investigated in this study. *E. coli* were isolated from 11.6% of urine, 7.4% of feces, 6.2% of HVS, and 7% of wound **Fig. 1**. In total, 355 isolates of Gram-negative bacteria comprising *E. coli* (n=173), *K. pneumoniae* (n=120) and *P. aeruginosa* (n=62). Antimicrobial resistance is a current public health menace of this century, warranting effective surveillance and monitoring protocol to assuage the evolution and transmission

of AMR bacteria within the hospital and general environments.

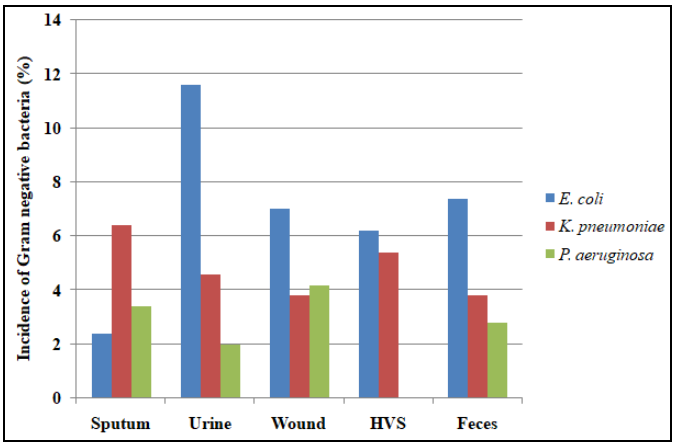

FIG. 1: PREVALENCE (%) OF GRAM-NEGATIVE BACTERIA IN THE CLINICAL SAMPLES INVESTIGATED

The Gram-negative bacteria of *E. coli*, *K. pneumoniae* and *P. aeruginosa* investigated in this study account for a handful of infections responsible for daily hospital visits in Nigeria, and these are also responsible for some community-acquired infections in the region. The prevalence of colistin-resistant bacteria in this study is shown in **Fig. 2**. A total of 41 *E. coli* strains (8.2%), 14 *K. pneumoniae* strains (2.8%) and 10 strains of *P. aeruginosa* from urine samples were colistin resistant. For wound samples, colistin resistance was not confirmed in the *K. pneumoniae* strains but 19 strains of *P. aeruginosa* (3.8%) and 24 strains of *E. coli* (4.8%) were confirmed as colistin resistant phenotypes **Fig. 2**. Since, no *P. aeruginosa* strains was recovered from HVS samples, colistin resistance was confirmed in 24 strains of *E. coli* (4.6%) and 18 strains of *K. pneumoniae* (3.6%). For fecal samples, colistin resistance was detected in 5 *P. aeruginosa* strains (1.0%), 13 *E. coli* strains (2.6%), and 11 *K. pneumoniae* strains (2.2%). Finally, colistin resistance was detected in 19 strains of *K. pneumoniae* (3.8%), 5 *E. coli* strains (1.0%), and 8 strains of *P. aeruginosa* (1.6%).

This study confirmed a total of 210 Gram-negative bacteria as colistin-resistant phenotypes. Of the 210 colistin-resistant Gram-negative bacteria, a total of 93 strains (18.6%) were phenotypically confirmed to be ESBL-producing bacteria that gave positive results in DDST. The incidence of colistin resistance in this study was high among *E. coli* isolates (8.2%) from urine samples, and this was followed by colistin-resistant *E. coli* recovered from the wound, HVS and fecal and sputum samples. Colistin resistance was also high in the *K. pneumoniae* and *P. aeruginosa* isolates in which a resistance rate was in the range of 1-3.6%. Existence of such significant levels of colistin resistance among Gram negative bacteria of *E. coli*, *K. pneumoniae* and *P. aeruginosa* has been previously reported in China, in which colistin resistance was high amongst bacteria isolates recovered from both human and animal isolates [13, 17]. In some recent studies conducted in China and Switzerland, the resistance of Gram-negative bacteria to colistin as reported in our study was confirmed in members of the *Enterobacteriaceae* family including *K. pneumoniae* in clinical samples of patients in the hospital environment [14, 25].

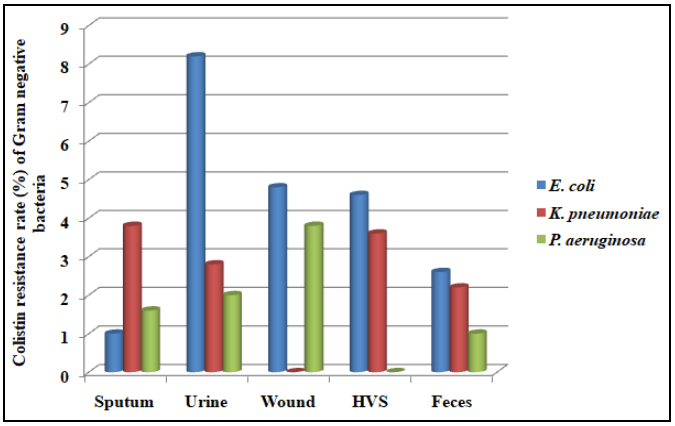

FIG. 2: PREVALENCE (%) OF COLISTIN RESISTANCE AMONG THE GRAM-NEGATIVE BACTERIA

This corroborates our reports of the growing incidence of colistin-resistant bacteria in hospital environments and warrants policy and surveillance to mitigate the evolution and spread of these drug-resistant strains of Gram-negative bacteria. **Table 3** shows the molecular characterization profile of ESBL and colistin-resistant genes in strains of Gram-negative bacteria isolated in this study. The most prevalent ESBL resistance genes detected were ^{bla}CTX-M genes that occurred in 4 strains of *E. coli*. The colistin resistance gene, *mcr-3* was also detected by PCR in 4 strains of *E. coli* **Table 3**. Both *mcr-1* and *mcr-3* was not detected in the *K. pneumoniae* strains. There was no detection of *mcr-2* gene in the *P. aeruginosa* strains investigated in this study. For ESBL genes, the ^{bla}SHV and ^{bla}TEM genes were not detected by PCR in the *K. pneumoniae* strains.

The prevalence of ESBL-producing bacteria was also investigated in this study. It was observed that the prevalence of ESBL among the tested bacteria was highest in the *E. coli* isolates, and this was followed in *K. pneumoniae* and *P. aeruginosa* in that order. In this study, only 93 strains of Gram-negative bacteria (18.6%) were phenotypically confirmed to be ESBL-producing bacteria. This corroborates with our previous study in which ESBL prevalence among Gram-negative bacteria was in the range of 8-20% [2, 4]. The ESBL genes investigated in this study were detected in the P. aeruginosa and E. coli strains except for K. pneumoniae strains, in which only the *bla*OXA and *bla*CTX-M ESBL genes were detected in two strains of *K. pneumoniae,* respectively. The *bla*TEM and *bla*SHV ESBL genes were not present in the *K. pneumoniae* strains that showed reduced susceptibility to the cephalosporins. However, the *bla*TEM and *bla*SHV ESBL genes were detected in 3 isolates of *E. coli*, respectively. In *P. aeruginosa* strains, the *bla*TEM ESBL gene was detected in only 2 *P. aeruginosa* isolates, while the *bla*SHV ESBL genes was detected in only one isolate of *P. aeruginosa*. In contrast, Surgers*et al.* [26] and Moglad, [27] reported a higher prevalence's of ESBL genes in members of the *Enterobacteriaceae*, including *E. coli* and *K. pneumoniae*, as well as in other bacteria such as *P. aeruginosa*. Though the reason for the disparities in the prevalence's of ESBL genes in this study is unclear, most studies have previously reported that these genes were responsible for resistance to the cephalosporins in Gram-negative bacteria [5, 7, 9].

TABLE 3: FREQUENCY OF ESBL AND COLISTIN RESISTANCE GENES IN THE GRAM-NEGATIVE BACTERIA

Resistance phenotype	Resistance gene	*P. aeruginosa*	*E. coli*	*K. pneumoniae*
ESBL	*bla*OXA	1	3	2
	*bla*CTX-M	3	4	2
	bla SHV	1	3	0
	*bla*TEM	2	3	0
Colistin	*mcr-1*	2	3	0
	mcr-2	0	2	1
	mcr-3	1	4	0

The results of the percentage susceptibility and resistance profiles of the ESBL and colistin resistant *E. coli, K. pneumoniae* and *P. aeruginosa* are shown in **Fig. 3**. Over 50% of the Gram-negative bacteria were found to be resistant to the tested antibiotics, particularly to colistin (100%), amoxicillin-clavulanic acid (100%) and ertapenem (100%) to which the *E. coli, K. pneumoniae* and *P. aeruginosa* were completely resistant to **Fig. 3**. High levels of resistance to the antibiotics were recorded in *P. aeruginosa* and *E. coli* which both showed reduced susceptibility to the cephalosporin sand fluoroquinolones **Fig. 3**. In no particular order, the *P. aeruginosa* isolates and *E. coli* strains showed high levels of resistance to ertapenem, nitrofurantoin, cefepime, ceftazidime and colistin that shows over 70% of the resistance. All the Gram negative bacteria were completely susceptible to imipenem (100%), while only *E. coli* strains showed complete susceptibility to gentamicin (100%), an aminoglycoside. Our results show that the prevalence of ESBL genes in *E. coli, K. pneumoniae* and *P. aeruginosa* may contribute a great deal to the incidence and distribution of cephalosporin resistance traits in Gram-negative bacteria. The colistin resistance genes detected in this study include *mcr-1-3*. *Mcr-1* gene was detected in 3 isolates of *E. coli* and 2 isolates of *P. aeruginosa*. None of the colistin-resistant *K. pneumoniae* strains harboured the *mcr-1* gene. For the *mcr-2* gene, only 2 isolates of *E. coli* and one strain of *K. pneumoniae* were confirmed to harbour the gene. The *mcr-3* gene was only detected in one isolate of *P. aeruginosa* and 4 isolates of *E. coli*. *mcr-3* gene was not detected in the *K. pneumoniae* isolates investigated in this study. Colistin resistance gene, including the *mcr 1-3* is responsible for the high prevalence of colistin resistant bacteria in the general environment [19]. The occurrence of colistin resistance genes (*mcr-1-3*) in our study corroborates previous studies that reported the evolution and distribution of mcr-1 positive E. coli and K. pneumoniae in human and animal samples [13, 25]. In a related study, *mcr-2* gene was recovered from *E. coli* isolates in Belgium [23]. The recovery of *mcr-3* positive colistin-resistant *P.*

aeruginosa strain in this study is the first report of a plasmid-mediated colistin-resistance gene in Southeast Nigeria. Gram-negative bacteria harbouring multidrug resistance genes such as ESBL and *mcr 1-3* are of public health importance because these antibiotic resistance genes can spur the evolution and transmission of drug-resistant strains of bacteria that may prove "too" difficult to treat. Based on the susceptibility testing of the ESBL and colistin-resistant Gram-negative bacteria, only imipenem, followed by gentamicin, had the best antimicrobial activity against the resistant isolates.

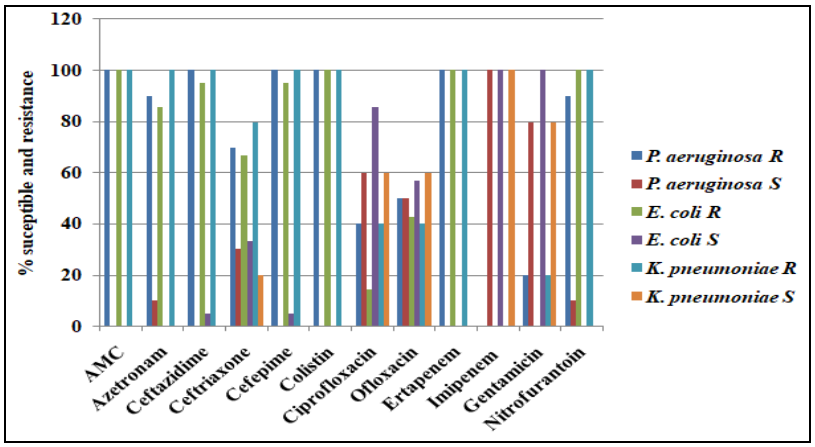

FIG. 3: RESISTANCE (%) AND SUSCEPTIBILITY (%) OF ESBL AND COLISTIN-RESISTANT BACTERIA. AMC =amoxicillin-clavulanic acid, R = resistance, S = susceptible

All the ESBL and colistin-resistant Gram-negative bacteria were completely susceptible to imipenem (100%). Studies have shown that carbapenems (e.g., imipenem) and a combination of other non-beta lactam agents (e.g., gentamicin) are usually the drugs of choice when an ESBL infection or a case of multidrug resistance is established [2, 7, 9]. It is important for hospitals to always be on the lookout for ESBL and colistin-resistant bacteria in patients' samples to guide therapy and reduce the transmission of such multidrug-resistant strains within the hospital environments. Finally, public awareness is required to imbibe rational use of antibiotics, and hospital staff retraining on detecting such multidrug-resistant bacteria is needed in this part of the world.

CONCLUSION: This study reports the recovery of ESBL and colistin-resistance genes in *E. coli, K. pneumoniae,* and *P. aeruginosa* from clinical samples in Abakaliki, Nigeria. The ESBL genes recovered were ^{bla}TEM, ^{bla}SHV, ^{bla}CTX-M, and ^{bla}OXA, while the colistin resistance genes isolated were *mcr 1-3*. This is the first report of *mcr-2* and *mcr-3* positive colistin-resistant Gram-negative bacteria in Southeast Nigeria. The isolation and characterization of Gram-negative bacteria harbouring such multidrug resistance genes (that mediates ESBL and colistin resistance in clinical isolates) indicate that both ESBL and colistin-resistant bacteria are spreading undetected in the general environment. Since infections caused by these multidrug-resistant strains are usually difficult to treat, it is necessary to monitor antibiotic use in the hospital and the general environment, particularly in agriculture and livestock production, where antibiotics are still under control in Nigeria.

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CONFLICTS OF INTEREST: The authors declare no conflicts of interest.

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