



## **Multiple-Antibiotic Resistance and Presence of CTX-M Genes among Enterobacteriaceae Isolates from Different Sources in Iwo, Osun State, Nigeria**

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### **Authors' contributions**

This work was carried out in collaboration between both authors. Author AOA carried out the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author OAA managed the analyses of the study, supervised the study and edited the manuscript. Both authors read and approved the final manuscript.

### **Article Information**

DOI: 10.9734/MRJI/2020/v30i930260

#### Editor(s):

(1) Dr. Mehdi Razzaghi-Abyaneh, Pasteur Institute of Iran, Iran.

#### Reviewers:

(1) Paola Vellante, Italy.

(2) Caroline Salezzi Bonfá, Federal University of the Jequitinhonha and Mucuri Valleys, Brazil.

Complete Peer review History: <http://www.sdiarticle4.com/review-history/62288>

**Original Research Article**

**Received 28 August 2020**

**Accepted 04 November 2020**

**Published 27 November 2020**

### **ABSTRACT**

**Aims:** Assessment of the antibiotic resistance pattern and detection of genes responsible for the  $\beta$ -lactam resistance in enterobacteria isolated from different sources was carried out in the course of the study.

**Place and Duration of Study:** Samples from different sources in Iwo, Osun State, Nigeria including Abattoir and Cow ranch. The study was done August 2015 to May 2016.

**Methodology:** Isolation was done on nutrient agar, xylose lysine deoxycholate (XLD) and identified using standard procedures. Antibiotic multidisc containing the following ceftazidime (30  $\mu$ g), cefuroxime (30  $\mu$ g), gentamicin (10  $\mu$ g), ciprofloxacin (5  $\mu$ g), ofloxacin (5  $\mu$ g), augmentin (30  $\mu$ g), nitrofurantoin (300  $\mu$ g), and ampicillin ( ) were used to study the resistance patterns of the bacteria. Polymerase chain reaction (PCR) method was carried out to screen for CTX-M gene in beta-lactam resistant isolates and statistical analyses were carried out using the analysis of variance test (ANOVA) and the PAST (paleontology statistics) software analytical package where applicable.

**Results:** Fifty-two (52) enterobacteria consisting of nine genera (9) were isolated and identified, *Serratia* (13), *Enterobacter* (11), *Klebsiella* (6), *E. coli* (5), *Proteus* (5), *Salmonella* (3), *Shigella* (3),

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*Citrobacter* (3), and *Yersinia* (3). The highest resistance observed by the isolates was to ampicillin (80.7%) followed by augmentin (40%) and 33% resistance of the isolates to cefuroxime, while all the isolates were sensitive to ceftazidime, gentamicin, ofloxacin, ciprofloxacin and nitrofurantoin. Nineteen of the isolates were resistant to three or more of the tested antibiotics i.e. multiple resistance while five of the multidrug resistant isolates show the presence of CTX-M  $\beta$ -lactam resistance gene which seems to be responsible for their resistance to the  $\beta$ -lactam antibiotic tested.

**Conclusion:** There are a number of organisms in the environment that have developed multiple resistances to several antibiotics and in view of this thwart treatment; of major concern are the Enterobacteriaceae family in which new resistance characteristics have been discovered leading to increasing resistance to standard available drug therapies.

**Keywords:** *B-lactam; 16s RNA enterobacteria; resistant genes.*

## 1. INTRODUCTION

During the last few decades, the incidence of microbial infections has increased dramatically. Continuous deployment of antimicrobial drugs in treating infections has led to the emergence of resistance among the various strains of microorganisms. Multiple-antibiotic resistance occurs when bacteria are resistant to more than one antibiotic and due to years of antibiotic overuse, it is now the rule rather than the exception among resistant bacteria [1]. This situation has largely occurred through the sequential use of multiple different antibiotics. The first antibiotic began by selecting a single resistance gene, eventually, however, bacteria resistant to the first antibiotic picked up resistance to others as they were introduced into the environment. The evolution of antibiotic resistance has been described as the most important evolutionary change in modern time, causing prolonged illness and increased cost of hospitalization for diseases that were once straightforwardly controlled [2,3,4].

Enterobacteriaceae species are important human pathogens. They can develop several mechanisms to avoid the inhibitory effect of antibiotics thus becoming resistant [2]. Enterobacteria are bacteria from the family Enterobacteriaceae, they are Gram-negative, rod shaped 1-3  $\mu\text{m}$  large bacteria, facultative anaerobes and their natural host is the human intestine where they are primarily known for their ability to cause intestinal upset. Examples include *Escherichia coli*, *Klebsiella* sp., *Proteus* sp., *Morganella* sp., *Providentia* sp., *Enterobacter* sp., *Serratia* sp. These bacteria can be pathogens of urinary tract, respiratory tract, bloodstream and wounds [5]. Their optimal growth temperature is between 22°C and 37°C; they grow on most simple bacteriological media and most can grow on D-glucose as the sole source of carbon, even though some require

amino acids and/or vitamins. Hence, they are able to use various carbohydrates and glucose both in the presence or absence of oxygen. Both acid and gas are usually formed from glucose. Enterobacterial species are oxidase negative and catalase positive [6]. They are responsible for a variety of human illnesses, including urinary tract infections, wound infections, gastroenteritis, meningitis, septicemia, and pneumonia. Several enterobacterial diseases are spread by fecal-oral transmission and are associated with poor hygienic conditions. Countries with poor water decontamination have more illness and death from enterobacterial infection.

This study was aimed at investigating the incidence of multiple-antibiotic resistance among Enterobacteriaceae isolates and to detect the genes responsible for  $\beta$ -lactamase resistance (CTX-M genes) in the multiple-antibiotic resistant isolates ( $\geq 3$ ) from different sources in Iwo, Nigeria.

## 2. MATERIALS AND METHODS

### 2.1 Sample Collection

Samples used for this research were collected from different sites (abattoir, poultry, cafeteria, hostel sewage, and cow ranch) in Iwo, Osun state, Nigeria. Different agar media were used in the course of the study which includes: nutrient agar, peptone water, *xylose lysine deoxycholate* (XLD) agar and Mueller-Hinton agar. All the media were prepared according to manufacturer's specification used for serial dilutions and streaking procedures for isolating the bacterial isolates.

### 2.2 Identification of Bacterial Isolates

Bacteriological characteristics were determined following available standard [7] and the characteristic features such as the phenotypic

and biochemical characteristics were studied to screen out non-enterobacteriaceae isolates.

### 2.3 Standardization of Inoculum and Antimicrobial Susceptibility Testing

The antimicrobial resistance or susceptibility profiles were evaluated by the agar disk diffusion method. The disk diffusion method used was carefully standardized and was performed according to the protocols of the Clinical and laboratory standards institute (CLSI) at the Biological Sciences laboratory, Bowen University. Four to five colonies were picked from an overnight growth on agar and inoculated into tryptone broth and incubated at 37°C for 18 hours. The inoculum was standardized to 0.5 McFarland standard, the turbidity was reduced and adjusted by adding sterile saline. The accuracy of the density of the McFarland standard was checked by measuring the absorbance (within 0.08-0.13) using a spectrophotometer at a wavelength of 625 nm and the adjusted inoculum suspension count of about 10<sup>8</sup> cfu/ml [8].

Antimicrobial susceptibility tests were performed using the Kirby-Bauer disk diffusion method on Mueller-Hinton agar plates using Gram negative antibiotic multidiscs (Rapid Labs, United Kingdom) containing ceftazidime (30 µg), cefuroxime (30 µg), gentamicin (10 µg), ciprofloxacin (5 µg), ofloxacin (5 µg), amoxicillin (30 µg), nitrofurantoin (300 µg), and ampicillin (10 µg). The antibiotic disks were applied to the inoculated plates and incubated at 37°C for 24 hours, the diameter of the zones of complete inhibition to the nearest whole millimeter was measured. Isolates that were resistant to three or more antimicrobials were considered to be multidrug resistant (MDR) [9].

### 2.4 Extraction of Genomic DNA, PCR Assay, Amplification of CTX-M Genes and Agarose Gel Electrophoresis

The multiple antibiotic resistant enterobacteria isolates (MDR ≥3) were sub-cultured onto fresh agar plates and grown overnight, genomic DNA were then extracted from the new bacteria growths according to [10].

PCR assays were performed to determine the presence of β-lactamase resistance genes in the MDR Enterobacteriaceae isolates according to [11]. Amplification of the DNA was performed in a PCR apparatus with *Taq* kit. All PCR experiments contained a positive control.

### 2.5 Statistical Analysis

The data obtained from this study was expressed in absolute values and in percentages. The geometric mean, median and mode of MIC values were determined using the Statistical SPSS package. Rates of antimicrobial resistance was compared among resistant bacterial genera using the PAST (Paleontology statistical) software. Percentage susceptibility and resistance of the isolates from the different sampling sites were represented by bar charts using the Microsoft Office Excel 2010 software package.

## 3. RESULTS AND DISCUSSION

The distribution of the isolated enterobacteria genera at the different sampling site is represented in Table 2. *Serratia* sp had the highest occurrence (13) followed by *Enterobacter* sp (11), *Klebsiella* sp (6). *E. coli* (5) and *Proteus* sp (5) had the same number of isolates followed by *Salmonella* sp (3), *Shigella* sp (3), *Citrobacter* sp (3), and *Yersinia* sp (3). Fig. 1. shows the cumulative percentage resistance of all the isolates to the tested antibiotics. The highest resistance observed by the isolates was to ampicillin (80.7%) followed by augmentin (40%) and 33% resistance of the isolates to cefuroxime. Out of the 52 isolates, 19 recorded multiple antibiotic resistance characteristics. A scatter plot (using the PLAST package) showing the isolates resistant to three or more antibiotics is presented on Fig. 2.

The gel electrophoresis of PCR-amplified *bla*-CTX-M gene from enterobacteria isolates is shown in Fig. 1 and Fig 2. Sixteen (16) isolates were screened namely 1(*Serratia*), 3(*Enterobacter*), 4(*Proteus*), 5(*Proteus*), 6(*Salmonella*), 8(*Klebsiella*), 9(*Enterobacter*), 10(*Enterobacter*), 11(*Proteus*), 13(*Enterobacter*), 14(*Serratia*), 15(*Enterobacter*), 16(*Proteus*), 17(*Klebsiella*), 18(*E. coli*) and 19(*Enterobacter*).

Fig. 1 shows the presence of CTX-M genes at 415bp on four isolates (isolates 3, 6, 14 and 16). These four isolates were observed at annealing temperatures of 60°C. The presences of multiple bands of different weights were also observed from the same bacterial isolates while Fig. 2 shows the presence of CTX-M gene at 415bp on isolates 13. This band was observed at a temperature of 61°C.

Today we can list a number of organisms in the community that thwart treatment because they

are resistant to not one, but many different antibiotics. The emergence of multiple antibiotic resistance is clearly related to the quantity of antibiotics and how they are being used [12]. Nine different Enterobacteriaceae genera isolated from different sampling sites during this study were subjected to different antibiotics. Many of the isolates exhibited high resistance to three of the antibiotics (ampicillin, augmentin, and cefuroxime) and very low resistance to four of the antibiotics (gentamicin, ciprofloxacin, ofloxacin and nitrofurantoin).

From the study *Proteus* isolates were highly resistant to cefuroxime, augmentin and ampicillin. *Salmonella* and *Shigella* were highly resistant to cefuroxime, while *Salmonella* isolates were also resistant to augmentin and nitrofurantoin. The *Enterobacter* isolates were also highly resistant to augmentin. The highest percentage resistance was observed to ampicillin with 80.7% cumulative resistance. Complete susceptibility was observed to ceftazidime (100%) followed by gentamicin and ofloxacin with 92% susceptibility as seen (susceptibility of the isolates to the antibiotics were significantly different at  $P = .05$ ).

The high resistance pattern by the isolated bacteria against ampicillin in this study supports a study conducted in Nigeria [2] where a high level of resistance to ampicillin was observed in Coliforms isolated from specific sites. Also in Abeokuta, Nigeria [13] where the antimicrobial susceptibility testing of commensal Enterobacteriaceae from free-range chickens using the broth micro-dilution method was done, 89.7% resistance to ampicillin was observed. High percentage resistance may be as a result of the various activities taking place at the sampling sites.

Among the fifty-two isolates in this study, *Serratia* constituted the largest portion (13), followed by *Enterobacter* (11), *Klebsiella* (6), *E. coli* and *Proteus* (5 each), *Salmonella*, *Shigella*, *Citrobacter* and *Yersinia* (3 each). The prevalence of *Serratia* may be as a result of activities (including human activities) in and around the different sampling sites. And the observed distribution of *Serratia* within the sampling sites shows its abundance in the environment.

Low percentage resistance was observed to gentamicin, ciprofloxacin, ofloxacin and

nitrofurantoin as all the enterobacter isolates were highly sensitive to these four antibiotics. Even though resistance to fluoroquinolones (ciprofloxacin) was not high in this study (10% resistance was observed), over the past decade the emergence of high-level, fluoroquinolone resistance among *E. coli* and other species of Enterobacteriaceae has been recorded [14].

The most effective antibiotic used in this study was ceftazidime followed by gentamicin, ciprofloxacin and ofloxacin, while augmentin and ampicillin were insignificantly different at  $P = .05$  to the clearing of the isolates, this finding is similar to those observed in Iwo, Nigeria [2] where the resistance pattern of Coliform was studied.

Nineteen of the isolates from this study were resistant to three or more antibiotics i.e. multiple antibiotic resistance (MAR), eight of which were from the abattoir, five from cow ranch, three each from cafeteria and poultry. Using PAST (Paleontological Statistics software), ampicillin is not seen on the scatter plot due to high resistance developed to it by the isolates. As observed, resistance to ampicillin by the Enterobacteriaceae isolates (80.7%) is not surprising. Semi-synthetic penicillins (e.g ampicillin and carbencillin) were introduced in the 1960s, generally they are now ineffective against some bacteria that are acquiring resistance, especially Enterobacteriaceae [15].

It can also be said from the PAST analysis that the isolates clustering around ciprofloxacin and ofloxacin have similar physical, chemical or genetic characteristic that made them react to some specific antibiotics in a similar way. Eight of these multidrug resistant isolates were from the abattoir, five from cattle ranch, three from poultry and three from the cafeteria. Presence of isolates with multidrug resistance at the abattoir was examined in a study in Nigeria [16], they examined the abattoir for bacteria with potential risk to human health, and it was found that several bacteria such as, *Escherichia coli* O157:H7, *Salmonella* sp. and *Campylobacter* sp. were present in the waste. Enterobacteria also have been isolated in effluent water from treating facilities at abattoirs [17]. A number of pathogens present in abattoir waste have their origin in digestive tracts of animals [18] and as such the spread of resistant bacteria and other foodborne pathogens within the environment is possible and of high concerns.

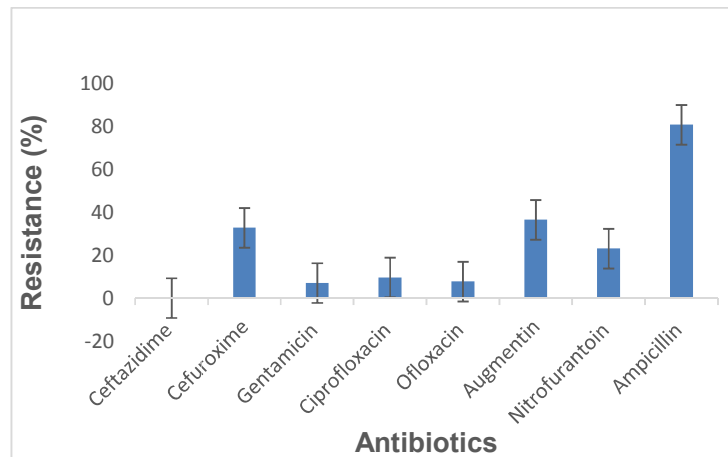


Fig. 1. Cumulative percentage resistance of isolates to tested antibiotics

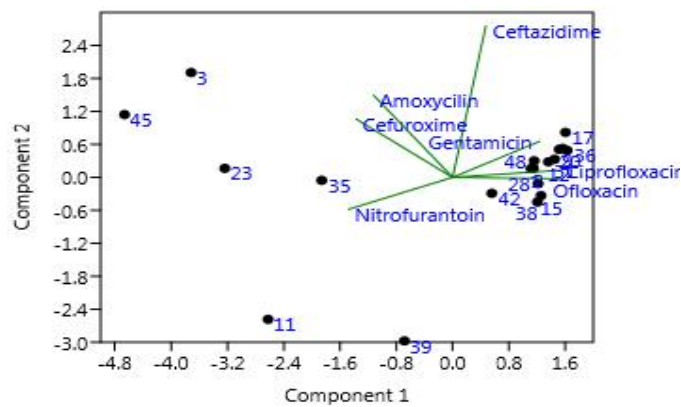


Fig. 2. Scatterplots of isolates resistant to three or more antibiotics

Table 1. Primer sequence used for PCR amplification

Primer	Sequence	Gene	Length of product (base pairs)
blaCTX-M1	Forward -5'- AAAAATCACTGCGCCAGTTC -3'	CTX-M	415
	Reverse- 3' -TTTTTAGTGACGCGGTCAAG -5'		
blaCTX-M2	Forward- 5'-AGCTTATTCATCGCCACGTT -3'	CTX-M	415
	Reverse- 3' -TCGAATAAGTAGCGGTGCAA -5'		

Resistance of Enterobacteriaceae bacteria to  $\beta$ -lactam antibiotic is mainly conferred by  $\beta$ -lactamases. The Bush-Jacoby-Medeiros classification grouped the  $\beta$ -lactamases in three major groups and sixteen subgroups. This classification is based on the substrates and inhibitors of the enzymes [19]. The most important  $\beta$ -lactamases are the cephalosporinases for example extended-spectrum  $\beta$ -lactamases (ESBLs) and the carbapenemases for example metallo- $\beta$ -lactamases (MBLs).

Resistance mechanisms against  $\beta$ -lactams are the outer membrane permeability change and efflux pumps [14]. ESBLs are  $\beta$ -lactamases capable of conferring bacterial resistance to the penicillins, early and extended-spectrum cephalosporins, and aztreonam (but not to cephamycins or carbapenems) by hydrolysis of these antibiotics, and are inhibited by  $\beta$ -lactamase inhibitors such as clavulanic acid, sulbactam and tazobactam. The most common ESBLs are SHV-, TEM and CTX-M [20].

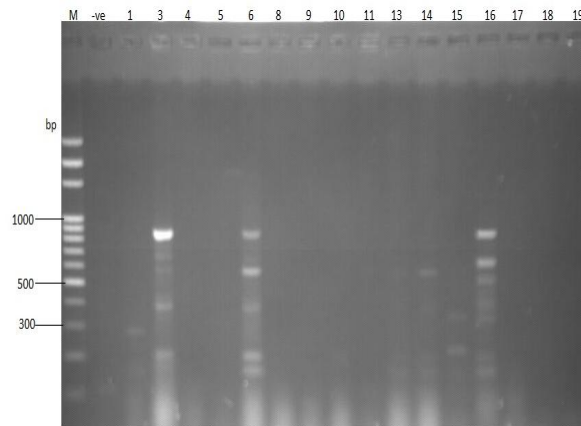
**Table 2. Distribution of isolated genera from the different sampling sites**

Sampling sites	Isolated Genera									Total
	<i>Serratia</i>	<i>Enterobacter</i>	<i>Klebsiella</i>	<i>E. coli</i>	<i>Proteus</i>	<i>Salmonella</i>	<i>Shigella</i>	<i>Citrobacter</i>	<i>Yersinia</i>	
Poultry	3	1	1	3	1	1	1	-	1	12
Hostel sewage	3	1	1	-	-	-	-	-	-	5
Cafeteria	1	3	1	2	1	1	-	-	1	10
Cow ranch	2	3	1	-	-	1	2	-	1	10
Abattoir	4	3	2	-	3	-	-	3	-	15
Total	13	11	6	5	5	3	3	3	3	52

In this study, PCR analysis was carried out to detect the presence of CTX-M gene in isolates resistant to three or more antibiotics. The gene was present and amplified in five of the multidrug resistance isolates tested as seen on Fig. 3 and Fig. 4. There are different reports on the prevalence of these enzymes in members of the Enterobacteriaceae family from different parts of the world [2,21,22,23].

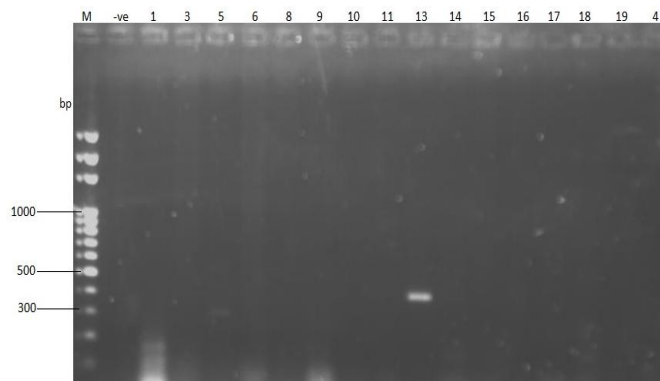
In some developing countries, including Nigeria, there is a high level of indiscriminate use of antibiotics both in humans and animals, [24] suggested that prolonged exposure to

therapeutic doses of these antibiotics (in plants, animals and humans) is the primary cause of antibiotic resistance. Therefore, factors such as levels of dependence on antibiotic usage, degrees of environmental pollution and prevailing climatic conditions from region to region may contribute to antibiotic selection pressure leading to the emergence of resistant bacteria, and the exposure of different hosts to these bacteria. According to the Clinical Laboratory Standards Institute [8], the use of antibiotics in food animals could enhance the development of antibiotic resistance and its transfer to human pathogens.



**Fig. 3. Agarose gel electrophoresis of PCR- amplified CTX-M genes (at 60°C annealing temperature) from beta-lactamase resistant Enterobacteria isolates**

Lane 1: Marker, Lane 2: negative control, Lane 3: *Serratia* sp (1), Lane 4: *Enterobacter* sp (3), Lane 5: *Proteus* sp (4), Lane 6: *Salmonella* sp (6), Lane 7: *Klebsiella* sp (8), Lane 8: *Enterobacter* sp (9), Lane 9: *Enterobacter* sp (10), Lane 10: *Proteus* sp (11), Lane 11: *Enterobacter* sp (13), Lane 14: *Serratia* sp (14), Lane 15: *Enterobacter* sp (15), Lane 16: *Proteus* sp (16), Lane 17: *Klebsiella* sp (17), Lane 18: *E. coli* (18) and Lane 19: *Enterobacter* sp (19)



**Fig. 4. Agarose gel electrophoresis of PCR- amplified CTX-M genes (at 61°C annealing temperature) from beta-lactamase resistant Enterobacteria isolates**

Lane 1: Marker, Lane 2: negative control, Lane 3: *Serratia* sp (1), Lane 4: *Enterobacter* sp (3), Lane 5: *Proteus* sp (4), Lane 6: *Salmonella* sp (6), Lane 7: *Klebsiella* sp (8), Lane 8: *Enterobacter* sp (9), Lane 9: *Enterobacter* sp (10), Lane 10: *Proteus* sp (11), Lane 11: *Enterobacter* sp (13), Lane 14: *Serratia* sp (14), Lane 15: *Enterobacter* sp (15), Lane 16: *Proteus* sp (16), Lane 17: *Klebsiella* sp (17), Lane 18: *E. coli* (18) and Lane 19: *Enterobacter* sp (19)

The development of MAR is a complicated issue which has become an international concern posing a serious menace to public health worldwide. To decrease the rise and spread of MAR, cooperative efforts are required because diseases which were curable earlier are becoming major causes of deaths [25]. Improved knowledge of molecular mechanisms controlling MAR should also facilitate the development of novel therapies to combat these intransigent infections.

With the help of molecular techniques,  $\beta$ -lactam resistance gene was tested for in this study as  $\beta$ -lactam drugs are one of the drugs of choice for the treatment of many bacterial infections nowadays. Although it is still very effective, a level of resistance to the drug was recorded in some of the isolates tested hence the need for further genomic analysis which will involve a better understanding of the pathobiology of these microbes and the development of new and effective antibiotics.

#### 4. CONCLUSION

The presence of multiple antibiotic resistant enterobacteria in the community continues to be a major health concern and this would make the control of antibiotic-resistant bacteria difficult. It is therefore important to stop the indiscriminate use of antibiotics, practice adequate personal hygiene and develop new and effective therapies to replace old ineffective ones. All these will influence the overall prevalence of antibiotic resistant bacteria within an ecosystem.

#### COMPETING INTERESTS

Authors have declared that no competing interests exist.

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