

**Original Article****Open Access****Emergence of New Delhi metallo-β-lactamase-1 (NDM-1) producing Enterobacterales from water sources: an impending public health challenge in Adamawa-north senatorial zone, Nigeria***¹Tula, M. Y., ²Enabulele, O. I., ²Ophori, E. A., ²Okojie, R. O., and ³Joel, F.¹Department of Biological Science Technology, Federal Polytechnic Mubi, PMB 035 Mubi, Adamawa State, Nigeria²Department of Microbiology, Faculty of Life Sciences, University of Benin, Nigeria³Department of Microbiology, Adamawa State University Mubi, Adamawa State, Nigeria*Correspondence to: birtyty@gmail.com**Abstract:**

Background: The emergence of New Delhi metallo-beta-lactamase-1 (NDM-1) among Enterobacterales in water sources has raised a major public health concern and constitute critical threat to human health as these organisms exhibits high level of resistance to available potent antibiotics. The aim of this study is to detect the presence of NDM-1 gene among carbapenem resistant Enterobacterales (CRE) isolates from water sources.

Methodology: A total of 256 water samples were collected from randomly selected hand-dug wells (128 samples) and river/stream (128 samples) for each of dry and rainy seasons in four out of the five local government areas (LGAs) of Adamawa-north senatorial zone, Nigeria. The water samples were filtered using membrane filtration technique and the filters introduced into appropriate bacteriologic media for bacterial growth. The bacterial isolates recovered were identified by both phenotypic and molecular protocols. Phenotypic carbapenem (imipenem) resistance was determined by disc diffusion test, *bla*_{NDM-1} gene was detected by specific polymerase chain reaction (PCR) test, and plasmid DNA was extracted and electrophoresed by standard procedure.

Results: Of the 256 water samples analyzed for bacteria growth, 300 bacterial isolates of the order Enterobacterales were recovered. Of these, only 45 (12.6%) isolates were phenotypically resistant to carbapenem (imipenem) antibiotic and *bla*_{NDM-1} gene was detected in 30 (66.7%) of these. While *bla*_{NDM-1} gene was detected in all the isolates of *Klebsiella oxytoca*, *Klebsiella variicola*, *Enterobacter aerogenes*, *Enterobacter hormaechei*, *Enterobacter asburiae*, *Citrobacter freundii*, and *Morganella morganii* that were resistant to imipenem, other isolates harbored *bla*_{NDM-1} gene in varying proportion. Most of the isolates positive for *bla*_{NDM-1} also harbored R-plasmids.

Conclusion: Emergence of carbapenem resistance mediated by NDM-1 gene in Enterobacterales isolated from water sources constitutes an emerging public health challenge with potential transmission to humans, thereby complicating the treatment of infections caused by these resistant pathogens in man. As such, the urgent need for antimicrobial surveillance and stewardship is of utmost importance.

Keywords: Enterobacterales; NDM-1; carbapenem; water sources; Adamawa; Nigeria

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Émergence de New Delhi métallob-β-lactamase-1 (NDM-1) produisant des Entérobactéries à partir de sources d'eau: un défi de santé publique imminent dans la zone sénatoriale nord d'Adamawa, au Nigeria*¹Tula, M. Y., ²Enabulele, O. I., ²Ophori, E. A., ²Okojie, R. O., et ³Joel, F.¹Département de Technologie des Sciences Biologiques, École Polytechnique Fédérale Mubi, PMB 035 Mubi, État d'Adamawa, Nigéria²Département de Microbiologie, Faculté des Sciences de la vie, Université du Bénin, Nigeria³Département de Microbiologie, Université d'État d'Adamawa Mubi, État d'Adamawa, Nigéria*Correspondance à: birtyty@gmail.com

Résumé:

Contexte: L'émergence de la métallo-bêta-lactamase-1 de New Delhi (NDM-1) parmi les entérobactéries dans les sources d'eau a soulevé un problème majeur de santé publique et constitue une menace critique pour la santé humaine, car ces organismes présentent un niveau élevé de résistance aux puissants antibiotiques disponibles. Le but de cette étude est de détecter la présence du gène NDM-1 parmi les isolats d'entérobactéries résistantes aux carbapénèmes (CRE) provenant de sources d'eau.

Méthodologie: Un total de 256 échantillons d'eau ont été prélevés dans des puits creusés à la main (128 échantillons) et des rivières/ruisseaux (128 échantillons) sélectionnés au hasard pour chacune des saisons sèches et pluvieuses dans quatre des cinq zones de gouvernement local (LGA) d'Adamawa-zone sénatoriale nord, Nigeria. Les échantillons d'eau ont été filtrés à l'aide de la technique de filtration sur membrane et les filtres ont été introduits dans des milieux bactériologiques appropriés pour la croissance bactérienne. Les isolats bactériens récupérés ont été identifiés par des protocoles phénotypiques et moléculaires. La résistance phénotypique au carbapénème (imipénème) a été déterminée par un test de diffusion sur disque, le gène *bla*_{NDM-1} a été détecté par un test de réaction en chaîne par polymérase (PCR) spécifique et l'ADN plasmidique a été extrait et soumis à une électrophorèse selon la procédure standard.

Résultats: Sur les 256 échantillons d'eau analysés pour la croissance bactérienne, 300 isolats bactériens de l'ordre Enterobacterales ont été récupérés. Parmi ceux-ci, seuls 45 (12,6%) isolats étaient phénotypiquement résistants à l'antibiotique carbapénème (imipénème) et le gène *bla*_{NDM-1} a été détecté dans 30 (66,7%) d'entre eux. Alors que le gène *bla*_{NDM-1} a été détecté dans tous les isolats de *Klebsiella oxytoca*, *Klebsiella variicola*, *Enterobacter aerogenes*, *Enterobacter hormaechei*, *Enterobacter asburiae*, *Citrobacter freundii* et *Morganella morganii* qui étaient résistants à l'imipénème, d'autres isolats abritaient le gène *bla*_{NDM-1} dans différentes proportions. La plupart des isolats positifs pour *bla*_{NDM-1} hébergeaient également des plasmides R.

Conclusion: L'émergence de la résistance aux carbapénèmes médiée par le gène NDM-1 chez les entérobactéries isolées des sources d'eau constitue un défi de santé publique émergent avec une transmission potentielle à l'homme, compliquant ainsi le traitement des infections causées par ces pathogènes résistants chez l'homme. À ce titre, le besoin urgent de surveillance et de gestion des antimicrobiens est de la plus haute importance.

Mots clés: Enterobacterales; NDM-1; carbapénème; sources d'eau; Adamawa; Nigeria

Introduction:

The order Enterobacterales consist of a large group of Gram-negative bacteria that are found in the large intestine of humans and other warm-blooded animals, mostly as endogenous microbiota. Implicated mostly in community and hospital-acquired infections, the most common therapeutic option for infections involving Enterobacteriaceae was the β -lactam antibiotics. However, with the emergence of multi-drug resistant Enterobacteriaceae, carbapenem, a broad-spectrum antibiotic became an ideal and last line of therapeutic option for the treatment of infections involving them (1). Many mechanisms for carbapenem resistance have been documented. The most prominent among them is the production of different classes of carbapenemases (2,3).

Carbapenemases are classified as β -lactamase enzymes that belong to Ambler molecular classes A, B and D (2,4) and are able to hydrolyse the entire β -lactam antibiotics, including monobactams, extended spectrum cephalosporins and carbapenem (2,3). Among the most reported carbapenemases is the New Delhi metallo- β -lactamase-1 (NDM-1), which is encoded by carbapenem resistance determinant, *bla*_{NDM} (5). The development and introduction of antibiotics for therapy came with a lot of prospects and hope. However, the occurrence of new resistant markers, notably NDM-1, incapacitated

the potentials of β -lactams as sure therapeutics for infections involving organisms that harbored such resistance markers. New antibiotic resistance markers are evolving every now and then due to mutation (6) and selective pressure in the use of antibiotics which consequently constitute a threat to therapy (4,7).

New Delhi metallo- β -lactamase-1 (NDM-1), a relatively newly described metallo- β -lactamase (MBL), can hydrolyze all β -lactams including carbapenems except monobactam. It was first identified in *Klebsiella pneumoniae* and *Escherichia coli* isolated from a Swedish patient who was hospitalized in India in 2008 (8). Since then, it has spread all over the world (9-11). Members of the order Enterobacterales harboring *bla*_{NDM-1} constitute clinical and public health significance as the gene encoding this enzyme is found on transmissible plasmid, as such resistance traits can be easily transferred from one bacterium to another (12,13).

The detection of *bla*_{NDM-1} gene in pathogens from water sources (14,15) and other environmental samples (16,17) in some parts of the world suggest that this gene is not only limited to clinical pathogens but is also present in our local environment (12). Moreso, the presence of antibiotic resistance genes in water sources meant for domestic purposes could serve as a vital reservoir for the spread of antibiotic resistance to human pathogens (18). Thus, this study intends to bring to limelight the

presence of *bla*_{NDM-1} gene among CRE from water sources in a pristine environment devoid of carbapenem usage.

Materials and method:

Study area:

The study area was Adamawa-north senatorial zone commonly known as Mubi region. Mubi region comprises of five local government areas (LGAs) namely; Madagali, Michika, Mubi south, Mubi North and Maiha with a land size of 4,493.815 km² and a population of 682,026. Mubi zone is located between latitudes 9° 30' and 11° 00'N of the equator and between 13° 00' and 14° 00'E of the Greenwich Meridian. The area has a tropical wet and dry climate, the dry season last for minimum of six months (November to March), while the wet season spans between May and October. The mean annual rainfall ranges from 700 to 1050mm. Some of the major ethnic groups in the region include Fali, Gude, Fulani, Marghi, Kilba, Nzanyi, Mudang, Zilwo, among others (19).

Sampling plan:

From each ward, water from four wells were chosen at random and sampled in duplicate in both dry and rainy seasons. A river/stream was also selected from each LGA for sampling. For each river/stream, two samples were collected at random (at upstream and downstream) in quadruples for both dry and rainy seasons.

Sample collection:

A total of 256 water samples from two sources (well and river) were analysed for bacterial growth. From these, 128 water samples were from 32 hand dug wells (64 water samples each for dry and rainy season) and another 128 samples were from 4 rivers/streams (64 water samples each for dry and rainy season). Each water sample was collected in 500ml sampling bottles, labelled appropriately and transported in an ice-cold box to the Microbiology Laboratory of the Department of Biological Science Technology, Federal Polytechnic Mubi, Adamawa State for analysis (20).

Isolation and identification of bacterial isolates:

Bacteria were isolated by membrane filtration technique using, a sterile 47mm, 0.45µm mixed cellulose ester (MCE) membrane filter (Merck, Bangalore). At the end of the filtration, sterile forcep was used to pick the filter unto the surface of MacConkey agar (MCA) and replicated on Salmonella-Shigella agar (SSA), eosin methylene blue (EMB) and deoxycholate citrate agar (DCA). The plates were incubated at 35-37°C for

18-24 hours.

After Gram stain, each discrete bacterial colony was identified to species level by biochemical tests such as Simmon's citrate test, triple sugar iron (TSI) agar and oxidase test before they were confirmed with Microgen GN A kit and 16S rRNA gene sequencing. The identified bacterial colonies were sub-cultured and stored in nutrient agar slant for further use.

Screening for carbapenemase production:

The isolates were screened for carbapenem resistance and hence possible carbapenemase producers according to CLSI guidelines (21). In this method, 10 µg imipenem antibiotic discs (Oxoid, UK) were placed on the surface of Mueller Hinton Agar (MHA) (Oxoid, UK) plates inoculated with each of the isolate and then incubated at 37°C for 24 h. After incubation, the zone of inhibition ≥ 23 mm in diameter was considered sensitive while the zone of inhibition ≤ 19mm in diameter was taken as resistance and was considered as suspected carbapenemase producers (21,22).

Molecular detection of New-Delhi metallo-beta-lactamase (*bla*_{NDM-1}) gene:

Phenol chloroform method was used for DNA extraction according to manufacturer's instructions (ThermoFisher Scientific). Dried extracted DNA was dissolved in 50 µl of DNase-free water and kept at -20°C for further use. Moreover, 5 µl of each DNA sample was checked for integrity on 1% agarose gel (23).

The conventional polymerase chain reaction (PCR) was used to amplify genes encoding NDM carbapenemase. Specific primer set (F-GGGCAGTCGCTTCCAACGGT, R-GTAGTGCTCAGTGTCGGCAT) targeting 475 bp was used to detect *bla*_{NDM-1} coding gene in 45 bacterial isolates resistant to imipenem in separate PCR reactions (24). PCR profile included an initial denaturing 5 min at 94°C, then 35 cycles of 94°C for 50s, 62°C for 45s and 72°C for 60s then terminate at 72°C for 10mins. The PCR was carried out in a Gene Amp 9700 PCR System Thermocycler (Applied Biosystem Inc., USA) using the appropriate profile as designed for each primer pair.

Five microlitres (5µl) of the PCR product was electrophoresed in 2% agarose gel containing 5 µl of 10 mg/ml ethidium bromide and ran at 100V for 45mins. A 1 kb plus DNA marker was used as molecular size marker. The PCR amplicons were visualized under ultraviolet (UV) transilluminator in a gel documentation system (25,26).

Plasmid DNA isolation:

The QIAGEN Plasmid Purification mini kit was employed to isolate plasmid DNAs from the

selected bacterial isolates (27) on the principles of alkaline lysis method (28).

Data analysis:

Simple percentage was used to tabulate the frequencies of the Enterobacterales

Results:

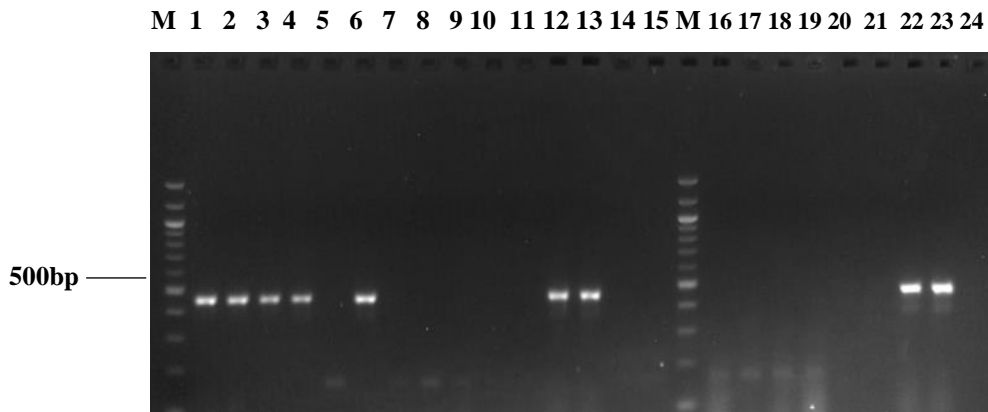
From the 256 water samples analyzed for bacteria growth, 300 enteric bacterial isolates belonging to two families (Enterobacteriaceae and Morganellaceae), 7 genera and 14 species were identified. Of these, only 45 isolates were phenotypically resistant to carbapenem

(imipenem) antibiotic accounting for 15.0% prevalence rate.

From these 45 isolates, *bla*_{NDM-1} was detected in 30 accounting for prevalence of 66.7%. *bla*_{NDM-1} gene was detected in all the isolates of *K. oxytoca*, *K. variicola*, *E. aerogenes*, *E. hormaechei*, *E. asburiae*, *C. freundii*, and *M. morgani* that were phenotypically resistant to imipenem. *bla*_{NDM-1} was also detected in *K. pneumoniae* (66.7%, 4/6), *E. coli* (50.0%, 6/12), *E. gergoviae* (50.0%, 1/2), and *P. mirabilis* (50%, 2/4) that were phenotypically resistant to imipenem (Table 1). Moreover, most of the isolates (63.3%, 19/30) carrying *bla*_{NDM-1} also harbored R-plasmids (Table 1, Plate 2).

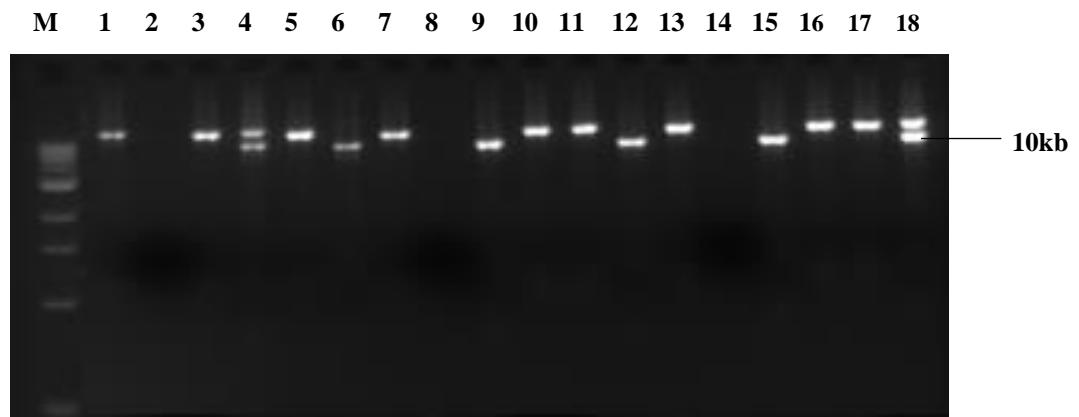
Table 1: Prevalence of NDM-1 gene among carbapenem (imipenem) resistant Enterobacterales

Bacterial species	No of isolates (%)	No of isolates resistant to imipenem (%)	No of imipenem-resistant isolates with <i>bla</i> _{NDM-1} (%)	No of <i>bla</i> _{NDM-1} positive isolates with R-plasmid (%)
<i>Escherichia coli</i>	63 (21.0)	12 (19.1)	6 (50.0)	2 (33.3)
<i>Klebsiella pneumoniae</i>	52 (17.3)	6 (11.5)	4 (66.7)	2 (50.0)
<i>Klebsiella oxytoca</i>	36 (12.0)	4 (11.1)	4 (100)	3 (75.0)
<i>Klebsiella variicola</i>	1 (0.33)	1 (100)	1 (100)	0
<i>Enterobacter aerogenes</i>	24 (8.0)	1 (4.2)	1 (100)	0
<i>Enterobacter cloacae</i>	30 (10.0)	2 (6.7)	0	0
<i>Enterobacter ludwigii</i>	1 (0.33)	1 (100)	0	0
<i>Enterobacter hormaechei</i>	11 (3.7)	3 (27.3)	3 (100)	3 (100)
<i>Enterobacter asburiae</i>	2 (0.7)	1 (50.0)	1 (100)	1 (100)
<i>Enterobacter gergoviae</i>	12 (4.0)	2 (16.7)	1 (50.0)	1 (100)
<i>Proteus mirabilis</i>	27 (9.0)	4 (14.8)	2 (50.0)	1 (50.0)
<i>Citrobacter freundii</i>	11 (3.7)	1 (9.1)	1 (100)	1 (100)
<i>Morganella morgani</i>	25 (8.3)	6 (27.3)	6 (100)	5 (83.3)
<i>Salmonella enterica</i>	5 (1.7)	1 (20.0)	0	0
Total	300	45 (15.0)	30 (66.7)	19 (63.3)



Band size approximately 475bp indicates the presence of the *bla*_{NDM-1} gene. Lane M= Molecular weight marker, Lane 1-4, 6, 12, 13, 22 and 23 are positive for *bla*_{NDM-1}. Lane 5, 7-11, 14-21, and 24 are negative for *bla*_{NDM-1}

Plate 1: Agarose gel electrophoresis of PCR products of *bla*_{NDM-1} gene in selected Enterobacterales isolates



M= Molecular weight marker. Lane 2, 8 and 14: No plasmid band. All other lanes have plasmid band. Lane 4 and 18 have 2 plasmid bands each. Lanes 1,3, 4, 5, 7, 10, 11, 13, 16, 17 and 18 have plasmid bands with MW >10kb

Plate 2: Agarose gel electrophoresis of R-plasmids in selected Enterobacterales isolates

Discussion:

Resistance to imipenem and discovery of the *bla*_{NDM-1} gene among imipenem-resistant Enterobacterales encountered in this study was unexpected. This is because this class of antibiotic was not in use in the entire study area during the period of carrying out this research. This was so significant because the study area was a pristine environment in which selective pressure may not have been responsible for the organism's resistance to carbapenem (imipenem) antibiotic and subsequent detection of the *bla*_{NDM-1} gene. A similar phenomenon was observed and reported by Adenipekun et al., (29) in Lagos although this was from clinical samples. According to the authors, carbapenem is rarely used in clinical practice in Nigeria except for emergencies or conditions that were critical, and

they concluded that detection of NDM-1 in Lagos may be connected to its geographical position; a border, vibrant and buoyant State that attracts all works of life from around the globe.

The observation that antibiotic resistance genes from bacteria are usually pervasive in natural environments, as well as places believed to be fallow (30,31), was evident in this study because the NDM-1 gene was discovered in an environment which was considered alien in the used of carbapenem. From the few data available in Nigeria, detection of *bla*_{NDM-1} gene was reported most often from clinical specimens (22,23). A study however reported the detection of *bla*_{NDM-1} gene from recreational beaches in Lagos (32). An additional study reported the detection of *bla*_{NDM-1} gene in pharmaceutical wastewaters from Lagos and Ogun States (16), while another study reported the detection of

*bla*_{NDM-1} from the soil of hospital environment in Akwa-Ibom State and Abuja, Federal Capital Territory, Nigeria (17). In Oghara, Delta State, Nigeria, *bla*_{NDM-1} was also detected in some water sources (33). In spite of these, there is paucity of information on prevalence of *bla*_{NDM-1} gene on surface and groundwater sources in Nigeria. As such, the finding of this study may serve as baseline data for epidemiological surveillance in the study area and the country at large.

*bla*_{NDM-1} gene code for the synthesis of an enzyme, New-Delhi metallo- β -lactamases-1 (NDM-1) that hydrolyses a broad range of antimicrobials, including carbapenems among others (8), which are last resort antibiotics for therapy of infections involving Gram-negative bacteria, especially ESBL-producing isolates (4). Carbapenem resistance may also be as a result of weak attachment of carbapenems to penicillin-binding proteins, and increasing expression of multidrug efflux pumps by the bacteria (34-36). Bacteria carrying *bla*_{NDM-1} gene are frequently called 'superbugs' because infections involving them are severe and most times not easily cured (18).

The detection of *bla*_{NDM-1} gene in pristine environment like ours and in water sources call for concern. This is because of the ability of organisms harboring NDM-1 to exhibit traits of multiple drug resistance. It has been reported previously that bacteria carrying NDM-1 gene tends to express, in addition to carbapenemases, other unrelated genes such as those encoding enzymes like ampicillinase C (AmpC), cephalosporinases, and modifying enzymes for aminoglycosides, macrolides, sulfamethoxazole, and fluoroquinolones (8,37). Moreso, because these water sources are often used for domestic and agricultural activities, the threat of transmission of 'superbug' to humans, as well as the transfer of NDM-1 encoding genes to strains of other Enterobacteriaceae species is imminent. Most importantly, pipe-borne water supply is unavailable in the entire study area and the available and dependable water sources were contaminated with 'superbugs' even before the introduction of antecedent antibiotics.

What would the future hold for these communities in terms of treatment option? This is so significant and worrisome because in Nigeria and other developing countries, antibiotic surveillance and stewardship is often relegated to the background. The problem is further compounded by the fact that most of the Enterobacterales isolates carrying the *bla*_{NDM-1} gene also harbored resistance-plasmids. This corroborates reports from previous studies (12,38,39). Studies have shown that the gene encoding

the production of NDM-1 enzyme is often localized on mobile plasmids which facilitates the dissemination of the gene rapidly between bacteria and different environments (13,40). Moreover, it was reported that plasmids harboring *bla*_{NDM-1} gene also bear several other resistance genes, thereby making these organisms superbugs (40), allowing limited or no therapeutic options (10). The extensive and inappropriate antibiotic usage in humans and animal husbandry including their subsequent spread into the ecosystem may have quickened the evolution, selection, and or the horizontal transmission of antibiotic resistance plasmids in bacterial populations as seen in this study (38).

Conclusion:

The findings from this study area shows the presence of Enterobacterales with plasmid-borne *bla*_{NDM-1} gene from water sources. Resistance to carbapenem antibiotics mediated by New Delhi metallo-lactamase-1 (NDM-1) constitutes an emerging challenge in the treatment of bacterial infections. As such, the urgent need for antimicrobial surveillance and stewardship is of utmost importance.

Contributions of authors:

MYT designed the study protocols and wrote the first draft of the manuscript. Authors OIE and EAO supervised and corrected the draft, while authors ROO and FJ gave professional and scientific advice, and also corrected the draft. All authors read and approved the final manuscript.

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Conflict of interest:

Authors declared no conflict of interest

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