Bangladesh J Med Microbiol 2018; 12 (2): 4-9
Bangladesh Society of Medical Microbiologists

Original Article

Acinetobacter baumannii: Identification, Antibiotic Sensitivity and Biofilm Formation in Different Clinical Samples

Maherun Nesa[1], Shaheda Anwar[2], Ahmed Abu Saleh[2]

[1]Department of Microbiology, Dhaka Medical Collage, [2]Department of Microbiology and immunology, Bangabandhu Sheikh Mujib Medical University

Abstract

Background: *Acinetobacter baumannii* is responsible for nosocomial infections which are related to biofilm formation of this pathogen. Biofilm formation helps the bacteria in surviving stressed environmental conditions and bacteria growing in biofilms are resistant to most of the commonly used antibiotics.

Objectives: The objective of this study was to detect *A. baumannii*, to see antibiotic sensitivity and biofilm formation in different clinical samples.

Methods: Total 108 *Acinetobacter* spp. were collected from different clinical samples which were identified by conventional microbiological procedures. Out of 108 *Acinetobacter* spp, 85 were identified as *A. baumannii* by polymerase chain reaction by detecting *blaOXA*-51 gene which is intrinsic to *A. baumannii*. Antibiotic sensitivity was detected by modified disc diffusion method and biofilm formation was detected by Tissue culture plate method.

Results: Among 85 isolates, 45.9% *A. baumannii* were obtained from tracheal aspirate followed by blood (21.2%), wound swab (15.3%), urine (10.6%), pus (5.9%) and pleural fluid (1.1%). More than 80% 0f *A. baumannii* was resistant to cephalosporin, aminoglycosides, quinolone, carbapenem. By Tissue culture plate method, 78.8% of isolates showed biofilm formation. Biofilm formation in tracheal aspirate was 82.1%, in blood 72%, in wound swab 92%, in urine 44.4%, in pus 100% and in pleural fluid 100%.

Conclusion: Detection rate of *A. baumannii* was more in tracheal aspirates. Biofilm producing *A. Baumannii* was resistant to most of the antibiotics.

Key words: Biofilm, Tissue culture plate method, Polymerase chain reaction

Introduction

Thirty-three genomic species (gen. sp.) of the *Acinetobacter* genus have so far been identified of which *Acinetobacter baumannii* is an important nosocomial pathogen that is responsible for a wide range of human infections such as pneumonia, septicemia, wound sepsis, urinary tract infections, endocarditis and meningitis.[1, 2] *A. baumannii*, genomic species 3 and 13TU, three of the most clinically relevant species, are genetically and phenotypically very similar to an environmental species, *Acinetobacter calcoaceticus*, and are therefore grouped together into the so-called *A. calcoaceticus–Acinetobacter baumannii* complex.[1] Because antibiotic susceptibility and clinical relevance are significantly different between different genomic species, exact identification of *Acinetobacter* species are required.[2] Identification within the genus is difficult and requires molecular methods.[3] Several genotypic methods have been developed for genomic species identification, which include amplified 16S rRNA gene restriction analysis, high-resolution fingerprint analysis by amplified fragment length polymorphism, sequence analysis of the 16S–23S rRNA gene spacer region, *rpoB* sequencing and *gyrB* multiplex PCR.[4] The *recA* gene[5] and *blaOXA-51*-like gene[5,6] were also used for *A. baumannii* genotypic identification. The genes *blaOXA-51*-like is intrinsically present in *A. baumannii*.[7]

Recently, the rapid development of multiple antibiotic resistance of *A. baumannii* has caused a serious problem for public health. The ability of biofilm formation contributes to *Acinetobacter* easily survive and transfer in the hospital environment, such as attached to various biotic and abiotic surfaces, e.g., vascular catheters, cerebrospinal fluid shunts or Foleys catheter.[8] In the clinical samples, the most commonly

Correspondence:
Dr. Maherun Nesa
Medical Officer
Department of Microbiology
Dhaka Medical College, Shahbagh, Dhaka-1000.
Tel : +8801740336877, Email : munbsmmu@gmail.com

encountered opportunistic pathogen is *A. baumannii* and because of its ability for colonization to the hospital setting and developing resistance, it leads to nosocomial infections that are difficult to treat.[9] Biofilm formation on invasive device by strains of *Acinetobacter* spp. is considered to be an important virulence factor for such infections.[10] Biofilm formation not only helps to protect the bacteria, but also helps in trading resistance genes between the participating cells. The ability of some *A. baumannii* isolates to produce biofilms might also explain its outstanding antibiotic resistance, survival properties and increased its pathogenicity.[11] This infection are difficult to eradicate as *Acinetobacter* spp. growing in biofilm are resistant to most of the antimicrobials thereby limiting therapeutic options. Biofilm formation on surfaces and expression of multidrug resistance favours dissemination of Acinetobacter spp. in hospital setting.[10]

Materials and Methods

This cross sectional study was conducted at the Department of Microbiology & Immunology, Bangabandhu Sheikh Mujib Medical University (BSMMU), Dhaka, Bangladesh from September 2017 - August 2018.

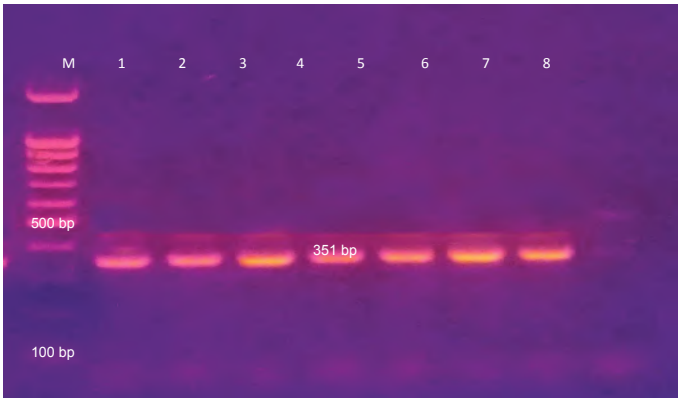
Bacterial Isolates and Laboratory Identification of *Acinetobacter* spp.

Total 108 *Acinetobacter* spp. were collected from different clinical samples (tracheal aspirate, wound swab, pus, blood, urine, pleural fluid). The clinical samples were sent from different ward of Bangabandhu Sheikh Mujib Medical University Hospital in Department of Microbiology & Immunology (BSMMU) for culture and antimicrobial sensitivity test. *Acinetobacter* spp. were identified by culture, Gram stain and biochemical tests (catalase test, oxidase test, urease test, motility test, citrate utilization test and Oxidation-Fermentation test).

Identification of *A. baumannii* by PCR

From 108 *Acinetobacter* spp. 85 were confirmed as *A. baumannii* by identification of the *blaOXA-51*-like gene. The PCR was performed in culture isolates using specific primers for detection of *blaOXA-51*-like gene (Applied biosystems, Thermofisher scientific, USA). DNA was extracted from bacterial colonies by boiling method. Two colonies of overnight growth of *A. baumannii* were taken in a 2 ml micro centrifuge tube (Extra-Gene, Taiwan). One ml of distilled water was added and boiled in a heat block (Incublock, Denville scientific inc. USA) for 10 minutes at 100° C. The tubes were then centrifuged for 5 minutes at 168 g (Hermle Z 233 M-2, Labnet international inc. USA). Supernatant was taken for PCR analysis.[12]

The specific primer for *blaOXA-51*-like gene was (Forward - 5′ TAA TGC TTT GAT CGG CCT TG; Reverse - 5'TGG ATT GCA CTT CAT CTT GG; Amplicon size 351 bp). The PCR assay was performed in a reaction mixture with total volume of 25 µl containing 15 µl of master mix (TBG biotechnology Corp. USA), 0.15 µl Taq polymerase (Solis BioDyne Germany), 1 µl of forward and reverse primer each (10 pmol/µl), 3 µl of distilled water and 5 µl of undiluted extracted DNA. The amplification condition was: initial denaturation at 95° for 5 minutes, 30 cycles containing of denaturation at 95° for 25 seconds, annealing at 60° for 40 seconds, extension at 72° for 50 seconds and final extension at 72° for 6 minutes. The amplified products were subjected to electrophoresis in 1.5% agarose gel.[6] (Photograph-I)



Antimicrobial Susceptibility Testing

A. baumannii were tested for antimicrobial susceptibility to ceftriaxone (30 µg), cefotaxime (30 µg), ceftazidime (30 µg), cefepime (30 µg), ciprofloxacin (5 µg), cotrimoxazole (1.25/23.75 µg), imipenem (10 µg), meropenem (10 µg), gentamicin (10 µg), amikacin (10 µg), netilmicin (30 µg), piperacillin-tazobactum (100/10 µg), ticarcillin-clavulanic acid (100/10 µg), colistin (10 µg), polymixin (300 units) (Oxoid, UK). The susceptibility was performed on Mueller Hinton Agar media (Merck, Germany) by modified disc diffusion method.[13] Zone of inhibition were interpreted per recommendation of the Clinical Laboratory Standard Institute (CLSI) guidelines.[14] In case of netilmicin zone of inhibition was used per BSAC standardized disc susceptibility testing method,[15] for colistin and polymixin B zone of inhibitions were used per Gales et al (2001).[16] *E. coli* (ATCC 25922) strains was used as the quality control reference strains according to CLSI.

Study of Biofilm Formation

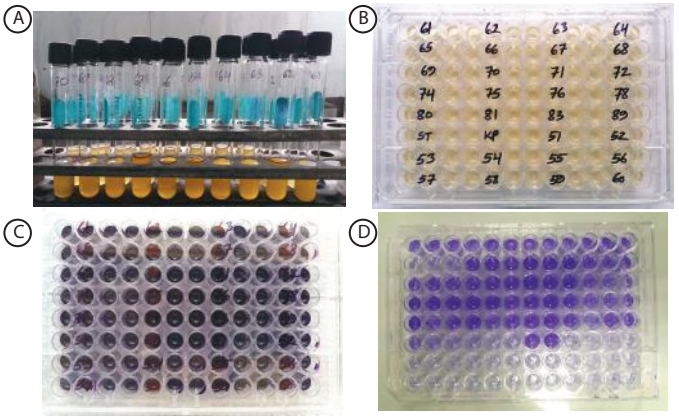
Biofilm production of *A. baumannii* was done by tissue culture plate method (TCP) according to Toledo-Arana et al (2001).[17]

A. baumannii were grown overnight in Brain heart infusion broth (BHIB) (Becton Dickinson and company, USA) with 0.25% glucose at 37°C. The broth culture was diluted at a ratio of 1:40. 200µl of this diluted culture suspension was inoculated in a sterile 96 well flat bottom polystyrene microtiter plate (Greiner Bio-One International, Kremsmunster, Austria). The positive control (*Klebsiella pneunoniae* ATCC 700603) and negative control (sterile BHIB-0.25% glucose) were also added

to microtiter plate in the same way. After overnight incubation at 37°C, the wells were gently washed three times with 200 µl of phosphate buffer saline (PBS). The plate was air dried, fixed with 200 µl/well of 2% formalin at 4ºC for 1 hour. After that, the wells were stained with 1% crystal violet for 15 min. Afterward, the wells were rinsed under running tap water to remove the excess stain. Then 200 µl ethanol-acetone (80:20, v/v) was added in each well to solubilize crystal violet. Each assay was performed in triplicate and repeated three times. The optical density (OD) at 630 nm was measured using ELISA plate reader (Plate reader, model–A4, serial no.-1910, Das, Italy).

The cut-off value (ODc) was calculated for each microtiter plate. ODc was of three standard deviations (SD) above the mean OD of the negative control: ODc = average OD of negative control + (3×SD of negative control).

Final OD value of a tested strain was expressed as average OD value of the strain reduced by ODc value (OD = average OD of a strain - ODc). If a negative value is obtained, it should be presented as zero, while any positive value indicates biofilm production.[18] Different steps of TCP method were depicted in photograph-II.



Data Analysis

The data were analyzed using SPSS software Version-23 (SPSS Inc., Chicago, IL, USA).

Results

Isolates and Identification

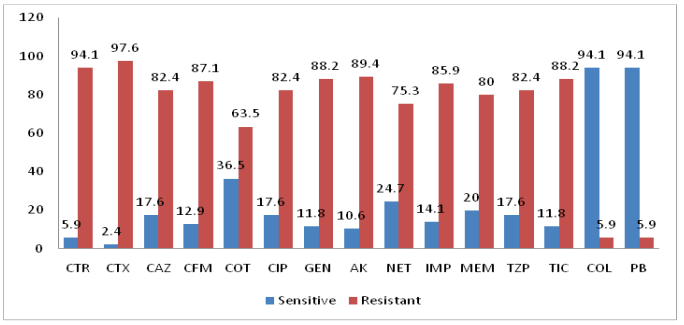
Total 108 *Acinetobacter* spp. were collected from which, 85 were identified as *A. baumannii*. Among 85 isolates, 39 (45.9%) *A. baumannii* were obtained from tracheal aspirate, 13 (15.3%) from wound swab, 5 (5.9%) from pus, 18 (21.2%) from blood, 9 (10.6%) from urine, 1 (1.1%) from pleural fluid. Majority of *A. baumannii* were isolated from tracheal aspirate (Table I).

Table-I: Distribution of *A. baumannii* Isolated from Different Type of Samples (n= 85)

Type of samples	Number of *A. baumanni* isolates	Percentage
Tracheal aspirate	39	45.9
Wound swab	13	15.3
Pus	05	5.9
Blood	18	21.2
Urine	09	10.6
Pleural fluid	01	1.2
Total	85	100

Antimicrobial Susceptibility of *A. baumannii*

Figure I showed antimicrobial resistance pattern of *A. baumannii*. *A. baumannii* showed 94.1%, 97.6%, 82.4%, 87.1%, 63.5% 82.4% 63.5%, 88.2%, 89.4%, 75.3%, 85.9%, 80%, 82.4%, 88.2% resistance to ceftriaxone, cefotaxime, ceftazidime, cefepime, cotrimaxazole, ciprofloxacin, gentamicin, amikacin, netilmicin, imipenem, meropenem, pipercillin-tazobactum, ticarcillin-clavulanic acid, colistin, polymixin B respectively.



CTR: Ceftriaxon, CTX: Cefotaxime, CAZ: Ceftazidime, CFM: Cefepime, COT: Cotrimoxazole, CIP: Ciprofloxacin, GEN: Gentamicine, AK: Amikacin, NET: Netilmicin, IMP: Imipenem, MEM: Meropenem, TZP: Pipercillin-tazobactum, TIC: Ticarcillin-clavulanic acid, COL: Colistin, PB: Polymixin B

Figure-I: Antimicrobial Resistance Pattern of *A. baumannii* (n=85)

Table II showed antimicrobial resistance pattern of *A. baumannii* in different type of samples. In tracheal aspirate, cefotaxime and ceftazidime were 100% resistant followed by ceftriaxone (97.4%), cefepime (97.4%), imipenem (97.4%), meropenem (97.4%), piperacillin-tazobactam (97.4%), ticarcillin-clavulanic acid (97.4%), amikacin (97.4%), ciprofloxacin (97.4%), gentamicin (94.7%), netilmicin (82.1%) whereas colistin and polymixin B showed only 2.6% resistance. In wound swab cefotaxime was 100% resistant followed by ceftazidime (92.3%), ceftriaxone (92.3%), cefepime (92.3%), amikacin

(92.3%), ciprofloxacin (92.3%), imipenem (84.6%), meropenem (84.6%), piperacillin-tazobactam (76.9%), gentamicin (84.6%). In blood cefotaxime was 88.9% resistant followed by ceftriaxone (83.3%), gentamicin (77.8%), amikacin (77.8%), netilmicin (72.2%). Pus, pleural fluid showed 100% resistance to all antimicrobial agent except colistin, polymixin B.

Table-II: Antimicrobial Resistance Pattern of *A. baumannii* in Different Type of Samples (n=85)

Antimicrobial agents	Type of samples					
	T/A (39) n (%)	W/S (13) n (%)	Pus (05) n (%)	Blood (18) n (%)	Urine(09) n (%)	PF (01) n (%)
Ceftriaxone (80)*	38 (97.4)	12 (92.3)	5 (100)	15 (83.3)	9 (100)	1 (100)
Cefotaxime (83)*	39 (100)	13 (100)	5 (100)	16 (88.9)	9 (100)	1 (100)
Ceftazidime (77)*	39 (100)	12 (92.3)	5 (100)	12 (66.7)	8 (89.9)	1 (100)
Cefepime (74)*	38 (97.4)	12 (92.3)	5 (100)	10 (55.6)	8 (89.9)	1 (100)
Cotrimoxazole (54)*	30 (76.9)	9 (69.2)	5 (100)	7 (38.9)	4 (44.4)	1 (100)
Ciprofloxacin (70)*	38 (97.4)	12 (92.3)	5 (100)	9 (50)	5(55.6)	1 (100)
Gentamicin (75)*	37 (94.8)	11 (84.6)	5 (100)	14 (77.8)	7(77.8)	1 (100)
Amikacin (76)*	38 (97.4)	12 (92.3)	5 (100)	14 (77.8)	6(66.7)	1 (100)
Netilmicin (64)*	32 (82.1)	8 (61.5)	5 (100)	13 (72.2)	5(55.6)	1 (100)
Imipenem (73)*	38 (97.4)	11 (84.6)	5 (100)	12 (66.7)	6(66.7)	1 (100)
Meropenem (68)*	38 (97.4)	11 (84.6)	5 (100)	7 (38.9)	6(66.7)	1 (100)
Piperacillin-tazobactum (70)*	38 (97.4)	10 (76.9)	5 (100)	9 (50)	7(77.8)	1 (100)
Ticarcillin-clavulanic acid (75)*	38 (97.4)	11 (84.6)	5 (100)	13 (72.2)	7(77.8)	1 (100)
Colistin (05)*	1 (2.6)	0 (0)	0 (0)	4 (22.2)	0 (0)	0 (0)
Polymixin B (05)*	1 (2.6)	0 (0)	0 (0)	4 (22.2)	0 (0)	0 (0)

Note: T/A: Tracheal aspirate; W/S: Wound swab; PF: Pleural fluid
*** Indicate resistant**

Biofilm Formation

Figure II showed that out of 85 *A. baumannii*, 78.8% produce biofilm and 21.2% were not produce biofilm.

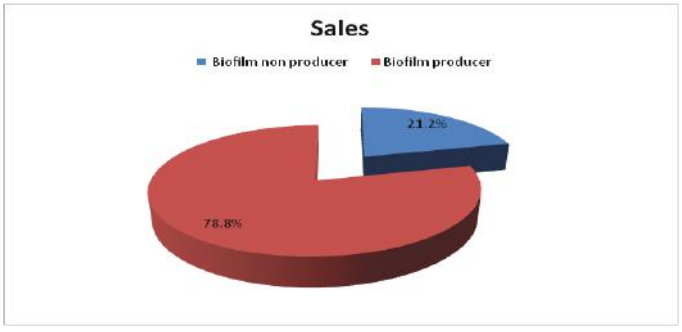


Figure-II: Biofilm production of *A. baumannii*

Table III showed Biofilm producer isolates of *A. baumannii* in different type of samples. *A. baumannii* showed 82.1%, 92.3%, 72.2%, 44.4% of biofilm formation in tracheal aspirate, wound swab, blood and urine respectively whereas showed 100% biofilm formation in Pus and pleural fluid.

Table-III: Biofilm producer isolates of *A. baumannii* in different type of samples

Type of samples	Biofilm producer isolates (67) n (%)	Non biofilm producer isolates (18) n (%)
Tracheal aspirate (39)	32 (82.1)	07 (17.9)
Wound swab (13)	12 (92.3)	01 (7.7%)
Pus (05)	05 (100)	0 (0)
Blood (18)	13 (72.2)	05 (27.8)
Urine (09)	04 (44.4)	05 (55.6)
Pleural fluid (01)	01 (100)	0 (0)
Total (85)	**67 (78.8%)**	**18 (21.2%)**

Discussion

Most of the *A. baumannii*, 45.9% of isolates were obtained from tracheal aspirates in this study. About 40.9% of *A. baumannii* in tracheal aspirates was reported by Barai et al (2010)[19] in Ibrahim Medical College, Dhaka. Angoti et al (2016)[20] reported 49.3% in Iran and Prabhu et al (2017)[21] found 20.4% *A. baumannii* in tracheal aspirate in Nepal. In this study 15.3% of *A. baumannii* was isolated from wound swab whereas Angoti et al (2016)[20], Babapour et al (2016)[22] isolated 11.6% and 10% of *A. baumannii* respectively. About 5.9% *A. baumannii* was isolated from pus in this study whereas 9.1% was isolated by Prabhu et al (2017).[21] In blood 21.2% of *A. baumannii* was isolated in this study and Angoti et al (2016)[20] isolated 16.7% of *A. baumannii*. About 10.6% of *A. baumannii* was isolated from urine in this study whereas 6.5% and 17% was isolated by Angoti et al (2016)[20] and Babapour et al (2016)[22] respectively. In pleural fluid 1.1% of *A. baumannii* was isolated in this study and Babapour et al (2016)[22] isolated 5% of *A. baumannii* in pleural fluid.

In this study, the resistance of *A. baumannii* isolates was found as follows: ceftriaxone (94.1%), cefotaxime (97.6%), ceftazidime (82.4%), cefepime (87.1%), cotrimoxazole (63.5%), ciprofloxacin (82.4%), gentamicin (88.2%), amikacin (89.4%), netilmicin (75.3%), imipenem (85.9%), meropenem (80%), pipercillin-tazobactum (82.4%), ticarcillin-clavulanic acid (88.2%). The resistance

rate of colistin and polymixin B was 5.9%. According to Prabhu et al (2017)[21] *A. baumannii* was found resistance against ceftriaxone (93.2%), cefotaxime (97.7%), ceftazidime (95.4%), cefepime (88.6%), ciprofloxacin (97.7%), cotrimoxazole (93.2%), gentamicin (52.3%), amikacin (43.2%), and piperacillin/tazobactam (97.7%). Angoti et al (2016)[20] found *A. baumannii* was resistance against ceftriaxon (99%), ceftazidime (98%), cefepime (99%), ciprofloxacin (99%), cotrimoxazole (84%), gentamicin (77%), amikacin (48%), imipenem (99%), meropenem (99%), colistin (11%). According to Babapour et al (2016)[22] 94.87% of the *A. baumannii* were resistant to Ceftriaxon, 94.23% to cefepime, 89.10% to cotrimoxazole, 83.33% to gentamicin, 91.03% to imipenem, 95.51% to Pipercillin-tazobactum, 93.59% to ticarcillin-clavulanic acid. There was a high degree of susceptibility to PB (300).

In this study, 67 (78.8%) of *A. baumannii* produce biofilm which was consistent with the results of Nahar et al (2013)[23] in Bangladesh, Gurung et al (2013)[10] in India, Thummepak et al (2016)[24] in Thailand. Nahar et al (2013)[23] and Thummepak et al (2016)[24] found 75% biofilm producing *A. baumannii*; Gurung et al (2013)[10] found 73% biofilm producing *A. baumannii*.

In this study, 82.1% of *A. baumannii* produce biofilm in tracheal aspirate whereas Gurung et al (2013)[10] and Rodríguez-Baño et al (2008)[25] reported 51.2% and 32% of biofilm formation in respiratory samples where they include tracheal aspirate and sputum. In this study biofilm formation was detected only in tracheal aspirate. This may be the cause of highest rate of biofilm formation in tracheal aspirate in this study. In blood, 72% biofilm producing *A. baumannii* was detected and 100% was detected by Rodríguez-Baño et al (2008).[25] About 44.4% of biofilm producing *A. baumannii* was detected in urine in this study whereas Gurung et al (2013)[10] and Rodríguez-Baño et al (2008)[25] reported 25% and 69% of biofilm producing *A. baumannii* in urine. 100% of biofilm producing *A. baumannii* was detected in pleural fluid in this study. Gurung et al (2013)[10] reported 100% biofilm producing *A. Baumannii* In sterile fluids (Pleural fluid and Peritoneal fluid).

Conclusion

This study demonstrated the ability of the clinical isolates of *A. Baumannii* to produce biofilm. Resistant to commonly used antibiotics such as cephalosporin, aminoglycosides, quinolone, carbapenem was also observed. Polymyxins were the only effective therapeutic agent in the study. This trend of multidrug resistance among *A. Baumannii* is a matter of concern.

Ethical Approval

Ethical clearance was taken from Institutional Review Board (IRB) of Bangabandhu Sheikh Mujib Medical University, Shahbag, Dhaka (No.BSMMU/2018/4348).

Funding

This work was supported by Department of Microbiology and Immunology, BSMMU and Research fund of Bangabandhu Sheikh Mujib Medical University (BSMMU).

Acknowledgments

The corresponding author is thankful to the Department of Microbiology and Immunology, Bangabandhu Sheikh Mujib Medical University (BSMMU) for providing necessary facilities to carry out the research.

Disclosure

The author reports no conflicts of interest in this work.

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