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Investigation of Quorum Sensing, Biofilm Production, and Detection of Virulence-associated Genes Among Clinical Isolates of *Acinetobacter baumannii*

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Abstract

Acinetobacter baumannii (A.baumannii) is an opportunistic microorganism able to survive in harsh environments and develop resistance to antibiotics often used in hospitals, which makes it one of the most common and pervasive causes of infections in healthcare facilities.The currentstudy was conducted to investigate the existence of*A.baumannii* and their antimicrobialresistantpattern, dispersion of Quorum Sensing(QS), and virulent related genes.In this investigation, the identification and susceptibility of 65non-repetitive *A. baumannii* isolates to antimicrobial drugs were evaluated. The QS encoding genes,and virulence-related genes were detected,and biofilm development was examined.In a total, 65 isolates of *A. baumannii*, 62 (95.38%) formed biofilm, with the vast majority being strong biofilm produces24(36.92%). The isolates showed a remarkable resistance to the antibiotics commonly used to treat *A. baumannii*as amikacin, which had a resistance rate of 100% among isolatesd.Results from virulence gene studies were as follows*csg*A (30.76%), *iut*A (24.61%), *cnfI* (16.92%), and *cva*C (6.15%). The two QS genes were *abal* (90.76%) and *abaR* (87.69%).Our findings confirm that the QS-related genes (*aba*I/*aba*R) were broadly dispersed across *A. baumannii* clinical isolates and were strongly associated with antibiotic resistance,and*csgA* was apredominant virulence gene followed by *itu*A.

Keywords*:* Antibiotic resistance, *Acinetobacter baumannii*,Biofilm, Virulence-related gene, Quorum sensing

1. Introduction

The opportunistic *Acinetobacter baumannii* causes a wide variety of clinical infections. Recently, it has come to light on a global scale, and it frequently leads to rise in antimicrobialineffectiveness and encoding of virulent-related genes(Zhang et al., 2022). Colicin V production (*cva*C), curli fibers (*csg*A), siderophores like aerobactin (*iut*A), and cytotoxic necrotizing factor (*cnf*I) are amongthe extremely important virulence-associated genes in*A. baumannii* isolates (Darvishi, 2016). Determination ofconcealed virulent-associated genes in clinical samples of *A. baumannii* has significant epidemiological repercussions that aid in controlling the spread of illnesses induced by this pathogen(Eraç et al., 2014, Eijkelkamp et al., 2014). *A. baumannii*capacity to acquire several virulence characteristics, particularly resistance determinants including efflux pumps, and iron acquirement pathways and motility, enables it to persist in hostile environments and promotes the occurrence of infections. This pathogen has been measured as a top member of nosocomial infections outstanding to its capacity to thrive in hospital conditions(Martinez et al., 2021).QS is a mechanism that bacteria use to accomplisha group of activities through sensing their population size, and constant production of small diffusible compounds known as autoinducers, such as N-acyl-homoserine lactones (AHLs) in some gram-negative pathogens(Gajdács and Spengler, 2019).The QS mechanism is widespread in bacteria and is associated with numerous biological mechanisms, such as locomotion, conjugation, biofilm development, synthesis of virulentfactors. The QS mechanism of *A. baumannii* consists of *aba*I/*aba*R, a two-component structure *aba*R gene encodes the receptor protein that adheres to AHLs and functions as a transcriptional regulator. The 3-hydroxy-C12-homoserine lactones tend to be the most common AHLs generated by the pathogen(Erdönmez et al., 2017).Main aims of this study include determining *Acinetobacter baumannii* from clinical samples and their antibiotic resistance profile, and detecting QS, virulence-associated genes.

2. Materials and Methods

2.1. Specimens collection

Between the 1st of October 2021 and the 30th of March 2022, 65 samples were collected from different sources, including sputum, wounds, and blood. Samples were collected from patients with impaired immunity at Erbil city, Kurdistan region-Iraq hospitals.

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2.2. Identification and antibiotic sensitivity testing

Identification of the isolates was accomplished using conventional microbiological techniques. The VITEK 2 GN card (BioMérieux, France) verified *A. baumannii* isolates. Antibacterial sensitivity testing on isolated bacteria was also accomplished using the compact automated system VITEK 2 to Pipracillin (PIP), Piperacillin/tazobactam (TZP), cefoperazone (CFP), ceftazidime (CAZ), Imipinim (IMP). Meropenem (MEM), Amikacin(AK), Gentamicin (GN),*Netilmicin* (NET), Tobramycin (TOB), Ciprofloxacin (CIP), Levofloxacin(LEV), Tagycycline (TGC), trimethoprim/sulfamethoxazole (SXT), colistin (CST).

2.3. A Carb NP protocol (CarbAcineto NP test)

CarbAcineto NP test was assessed asstated byDortet et al. (2014). *A.baumannii* clinical specimens were overnight cultivated in TSA. Two tubes were used (Tube A and B). One loop of the tested bacteria was resuspended by vortexing it for 15 seconds at maximum agitation in 100 µL of lysis buffer containing a 5.0 M NaCl solution. 100 µL of the phenol red-containing revealing solution was introduced to tube A; simultaneously, 100 µL of the same solution was introduced to tube B, including 6 mg/mL of imipenem. Both tubes were incubated at 37°C for two hour. Visual examination of the color shift in both tubes was recorded.

2.4. Motility characteristics

Swarming and twitching motility was evaluated using LB broth with 0.4% or 0.8% agar. Positive swarming motility was defined as a zone larger than 10mm surrounding the inoculation siteafter plates incubated at 37°C for 48 hours, For twitching motility, bacteria were dyed with a 0.2% crystal violet solution. Positive twitchers had zone diameters of 5mm or more. Each isolate was examined 3 times(Clemmer et al., 2011).

2.5. Biofilm detection by microtiter plate

Biofilm development capability was tested with crystal violet.After overnight incubation, bacteria were diluted to an optical density of 600 (OD_{600}) = 0.1. Then, 180 µL of Luria-Bertani (LB) broth and 20 µL of bacterial suspensions were put on 96-well polystyrene microtiter plates. The plates were incubated at 37°C for 24 hours and then washed three times with phosphate-buffered saline (PBS) and stained with 200 µL of 0.1% crystal violet (Oxoid, UK). Consequently, the plates were rinsed three times with PBS. The plates were dissolved in 200 µL of 95% ethanol by gently stirring at room temperature for 20 minutes. At 570nm, the absorbance was determined. Three standard deviations (SD) above the average OD of the negative control (LB broth only) were defined as the cut-off OD (ODc). The classification rules were as follows: OD≤ODC indicates non-biofilm producers (-); ODC<OD≤2×ODC indicates weak biofilm producers (+); 2×ODC<OD≤4×ODC shows moderate biofilm producers (++), and OD>4×ODC suggests strong biofilm producers (+++)(Li et al., 2021).

2.6. Genomic extraction fromA. baumannii isolates

Genomic DNA was isolated from a bacterial colony using a Fermentas Germany kit accordingtothe manufacturer's guidelines.Nanodrop was used to assess DNA concentration by spectrophotometer at 260 nm, then stored at –20°C until use(Darvishi, 2016).

2.7. Molecular detection of QS and virulence-related genes

Employing standard polymerase chain reaction (PCR), the existence of the QS and virulence-associated gene in all isolates was determined. The experiment was conducted in a 25 µL reaction mixture including 12.5 µL of 2× master mix (AMPLIQON, Denmark), 1.0 µL of each primer (10 pmol), and 1.5 µL of genome template. The amount reached 25 µL using PCR water. The following was cyclingconditions: First 1 cycle at 95°C for 4 min; thirty cycles at 95°C for 50 sec, 58°C for 60 sec, and 72°C for 45 sec; one cycle at 72°C for 8 min (Al-Kadmy et al., 2018a). The program for QS genes was as follows: 30 cycles of denaturation at 94 °C for 1 min, annealing at 52 °C for 30 s, and extension at 72 °C for 1.5 min, with a final elongation step at 72 °C for 5 min.occurrence of the QS and virulence-related genes were identified by applying the primer sequences of*aba*I 435bp and*aba*R 310bp (Tang et al., 2020). *cnf*I 498bp, *csg*A 200bp, and *iut*A 300bp (Al-Kadmy et al., 2018b) as shown in (Table.1).

Table 1. Primer sequences and size of PCR products for QS and virulence-associated genes.

Target genes	Oligonucleotide primers Sequences (5'-3')	Product size (bp)
***aba*I**	Forward:AAAGTTACCGCTACAGGG Reverse: CACGATGGGCACGAAA	435bp
***aba*R**	Forward:TCCTCGGGT CCCAATA Reverse: TAAATCTACCGCATCAA	310 bp
***cnf*I**	Forward:AAGATGGAGTTCCCTATGCAGGAG Reverse:CATTCAGAGTCTTGCCCTCATTATT	498 bp
***csg*A**	Forward:ACTCTGACTTGACTATTACC Reverse: AGATGCAGTCTGGTCAAC	200 bp
***cva*C**	Forward:CACACACAAACGGGAGCTGTT Reverse: CTTCCCGCAGCATAGTTCCAT	680 bp
***iut*A**	Forward:GGCTGGACATCATGGGAACTGG Reverse: CGTCGGGAACGGGTAGAATCG	bp

2.8. Statistical analyses

GraphPad Prism 9.01 was used for all statistical analysis. Data were evaluated using the X^2 test,$\chi 2$ test, or Fisher's exact test and Kruskal – Wallis test. The p-value ≤ 0.05 was used to identify the differences as statistically significant.

3. Results

3.1. QS and virulence-associated gene distribution among A. baumannii isolates

In this invistigation, sixty-five (65) isolates of*A. baumannii* were diagnoseddepending on colonial morphology on cultural media and various biochemical characteristicsthen confirmed by VITEK 2 system. The gender distribution of the isolates was as follows: 24 (36.92 %) for females and 41 for males (63.07%). Out of the 65 isolates, 59 (90.76 %) had the *abaI* QS gene, while *abaR* found in 57 (87.69 %),andthe virulence genes were

distributed as follows: *csgA* = 20 (30.76%), *iutA* = 16 (24.61%), *cnfI* = 11 (16.92%), and *cvaC* = 4 (6.15%) (Table.2).

Table 2. Distribution of QS genes and virulence-related genes among*A. baumannii isolates*.

Gene type	Gene function	Prevalence	
		n.	%
***aba*I**	Quorum sensing genes	59	90.76
***aba*R**		57	87.69
***csg*A**	Curli fiber adhesive virulence	20	30.76
***iut*A**	Sidrophore like aerobactin	15	24.61
***cnf*I**	Cytotoxic necrotizing factor	11	16.92
***cva*C**	Colicin V production	4	6.15

3.2. Resistance to antimicrobial agents

All isolates demonstrated complete resistance to amikacin. Followed by ciprofloxacin 95.38%, each of pipracillin tazobactum, ceftazidime, imipenem, and levofloxacin exhibited 93.85%. Colistin had the most significant efficiency towards *A. baumannii*, as all the isolates were sensitive to colistin(figure.1).

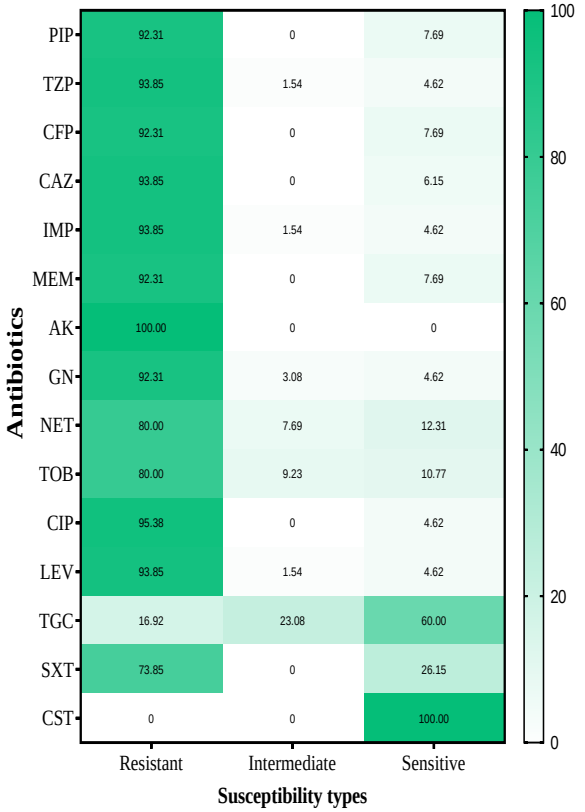

Figure 1. Antimicrobial resistance patterns in *A. baumannii*

3.3. CarbAcineto NP assay

The ability of carbapenemase production was assessed for 65 isolates using a CarbAcineto NP test. Forty (61.53 %) of the isolates tested showed positive results in the carbAcineto NP test. as shown in figure.2.

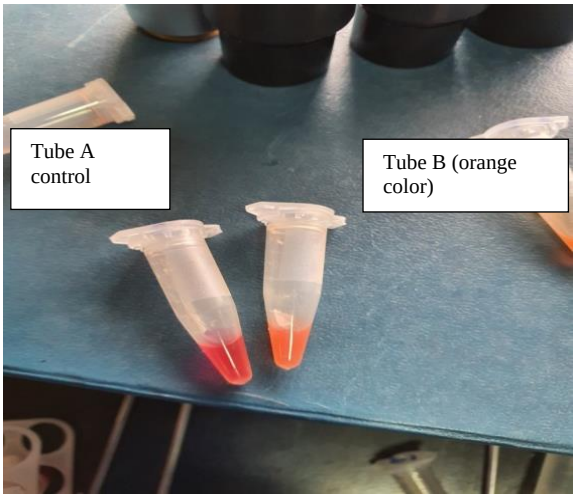

Figure 2. Craba NP test colour change in tube B to (orange colour) indicate positive result.

3.4. Motility characteristics and source of specimens

All of the isolated bacteria were tested for their motility. Surface-associated motility (swarming) was detected in 17 (26.15%), and twitching motility was observed in 26 (40%) of the *A. baumannii* isolates.As depicted in (*Figure. 3*), sputum isolates had superior twitching motility over wound and blood samples. 21/45 (46.6%) sputum isolates exhibited twitching movements. Similarly, the swarming motility among all isolates of *A. baumannii* recorded the highest frequency in sputum sources.Our findings revealed that the high prevalence of the swarming motility was in sputum 15/45 (33.33%), while the lowest incident of swarming motility was in the blood and wound specimens (*n* =1).

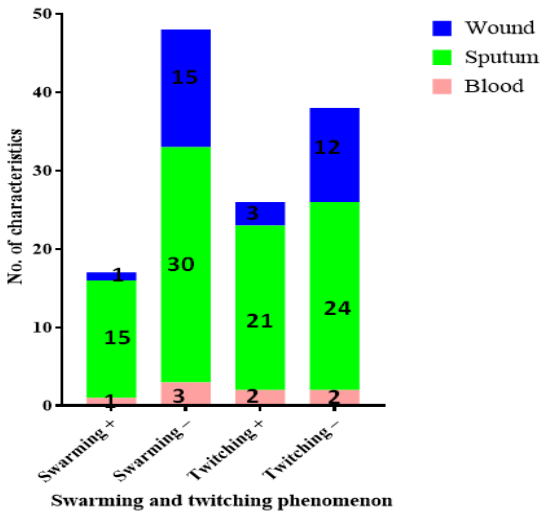

Figure 3. Prevalence of phenotypic swarming and twitching phenomenon among the source of specimens.

3.5. *Biofilm formation, QS, and virulence-related*

In terms of biofilm production, the results indicated that 62 (95.38%) isolates were able to form biofilms, of which 24 (36.92%) were strong biofilm producers, 18 (27.69%) and 20 (30.77%) were moderate and weak producers, respectively.

On the other hand, 3.6% of the isolates do not have the ability to develop biofilm. We diligently investigated the

association between biofilm development and genes associated with QS signaling molecules and virulence factors in *A. baumannii* isolates. A highly developed ability to form biofilms is demonstrated by isolates that possess the genes for QS signaling molecules (*abaI* and *abaR* genes), Additionally, a notable disparity was noted in comparison to the isolates lacking the QS-related genes (Figure. 4). Conversely, there was no significant correlationbetween the isolates that have the virulence-related genes to produce the biofilm and the isolates that do not bear the virulence-related gene.

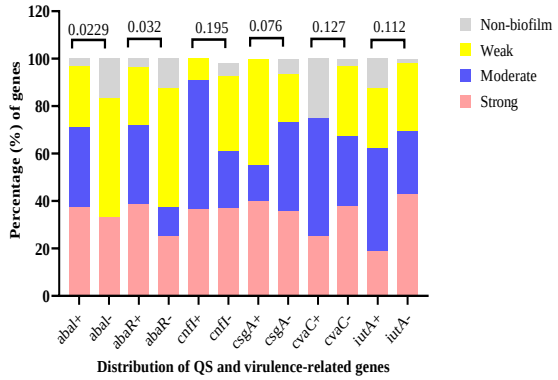

Figure4. Quorum sensing and virulence gene distribution across biofilm formation states

3.6. Association between virulence genes with antibiotic-resistant

Isolates that are resistant to antibiotics have higher number of QS and virulence genes compared to isolates that are susceptible. The prevalence of *cnf*I in isolates resistant to aminoglycosides (tobramycin and amikacin), imipenem, and meropenem was significantly higher than in comparable sensitive isolates. Among the great majority of antibiotic-resistant isolates, the proportion of csgA was much higher than susceptible isolates. The ratio of *cva*C across all antibiotic-resistant isolates was substantially greater than comparable susceptible isolates. The proportion of *iut*A in isolates resistant to ciprofloxacin, gentamicin, tobramycin, amikacin, imipenem, and meropenem was significantly greater than isolates susceptible to comparable antibiotics (Table.3).

Table 3. Existence of virulence-associated genes with antibiotic-resistant among*A.baumannii*.

	Antibiotics													
Genes	PIP	TZP	CFP	CAZ	IMP	MEM	AK	CN	NET	TOB	CIP	LEV	TGC	SXT
QS gene														
*aba*I (59)	91.5	93.2	91.5	93.2	93.2	91.5	100.0	91.5	76.3	78.0	93.2	18.6	71.2	71.2
*aba*R (56)	92.9	94.6	92.9	94.6	94.6	92.9	100.0	92.9	78.6	78.6	96.4	94.6	17.9	73.2
Virulence-Related Genes														
*cnf*I (11)	46.7	53.3	46.7	53.3	53.3	46.7	73.3	46.7	26.7	26.7	53.3	53.3	6.7	46.7
*csg*A (20)	90.5	90.5	90.5	85.7	90.5	90.5	95.2	90.5	85.7	85.7	95.2	90.5	14.3	81.0
*cva*C (4)	33.3	50.0	33.3	50.0	33.3	33.3	66.7	33.3	16.7	16.7	33.3	50.0	0.0	33.3
*iut*A (16)	80.0	85.0	80.0	85.0	80.0	50.0	100.0	80.0	60.0	70.0	85.0	85.0	20.0	70.0

3.7. Association of QS with antibiotic resistance

The frequency of antibiotic susceptibility pattern inisolates containing QS genes (*aba*I and *aba*R) is depicted in (Figure.5).54 of isolates expressing the *abaI* gene were resistant to antibiotics, while only 5 of isolates lacking the *abaI* gene were resistant to antimicrobials. Only 9 of the resistant isolates were found to be deficient in the *abaR* gene, while 51 of the isolates expressing the gene were antibiotic-resistant bacteria. Antibiotic resistance was found to be significantly associated with the presence of the *abaI* and *abaR* genes in the isolates (*p*-value = 0.002).

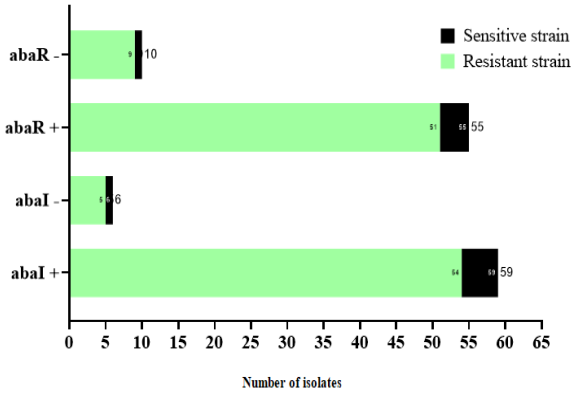

Figure 5. Association between antibiotic resistance and carrier position of QS genes.

4. Discussion

*Acinetobacter baumannii*is an opportunistic bacterium that can spread in healthcare facilities, potentially resulting in the emergence of nosocomial illnesses(Jahangiri et al., 2019, Nowak et al., 2014). Because of its adhesive properties, it poses a significant risk of colonization and transmission among hospitalized patients(Choi et al., 2010). This pathogen is able to withstand a wide range of modern antibiotics due to the acquisition of resistance determinants and the upregulation of intrinsic resistance pathways(Zeighami et al., 2019). Multidrug-resistance*A. baumannii*produces severe illness and significant mortality, particularly in immune-compromised individuals(Howard et al., 2012, Poirel et al., 2011).In the current study, several widely used antibiotics had no effect on the *A. baumannii* isolates.All were resistant to amikacin and sensitive to colistin, which is consistent with findings from other Iranian studies (Vahdani et al., 2011, Saffari et al., 2017). Even though all isolates were susceptible to colistin, colistin remains the most effective antimicrobial agent (Amin et al., 2019). In our study,90.76%and 87.69% of isolates were positive for each*abaI* and *abaR*respectively*;* we also found an association between antibiotic susceptibility and QS genes andrevealed that a greater number of resistance isolates carried both QS genes. This demonstrates that *aba*I and *aba*R genes were substantially linked with multidrug-resistant pathogenes, signifying that isolates having QS genes were more likely to be drug-resistant, although the pathogenicity and virulence factors of *A. baumannii* are not completely known. In terms of the incidence of virulence genes, *csg*A 20(30.76%) was the predominant once, and *cva*C 4(6.15%) had the lowest frequency.No correlation was found between virulence genes and biofilm production, but antibiotic susceptibilityisolates carried a greater number of virulence genes than antibiotic insusceptible isolates.The results of this study are consistent with those of an Iraqi investigation showing a maximum frequency of 66.7% for *csgA* and a lowest frequency of *cvaC* (9.5 %), Al-Kadmy et al. (2018a).Comparatively, another study indicated that *cvaC* had lowest frequency only 10% and *csgA* was 55%among tested isolates, Momtaz et al. (2015). Nevertheless, the findings of the current study contrasted withthose of two other studies, Darvishi (2016)and Nazari et al. (2021), whosehighest frequency was *cnf*1(35.53%), and the lowest occurence was*csg*A (12.39%).*A. baumannii* ability to form biofilms isalso largely depend on virulence factors(Rosales-Reyes et al., 2017, Sanchez-Larrayoz et al., 2017). The potential of *A. baumannii* to inhabit and formbiofilm on different surfaces is a crucial element in chronic and persistent infections(Thummeepak et al., 2016). According to our findings, 62(95.38%) *A. baumannii* isolates generated biofilm. Other studies found that greater than 75% of *A. baumannii* isolates develop biofilms, which agrees with our results (Thummeepak et al., 2016, Sung, 2018, Al-Shamiri et al., 2021). Our isolates exhibit a twitching form of motility more than a swarming form, and the majority of both formsof movement were from sputum source, which disagrees with (Loraine et al., 2020, Skerniškytė et al., 2019) who found both forms of motility from other source like blood. According to our finding carbaNP test was able to detect 61.53% of carbapenem producer.Similar findings were detected by other the studies(Khuntayaporn et al., 2021, Nguyen and Joshi, 2021). The use of carbapenems is the very last resort in the fight against illness induced by gram-negative multi-drug resistant pathogens; nonetheless, carbapenem insusceptiblity is becoming more prevalent in *A. baumannii*. Consequently, crucial and precise diagnosis of carbapenemase-producing *A.baumannii* is essential for the treatment effectiveness of these illness.

5. Conclusion

The propensity of *Acinetobacter baumannii* to form biofilms and its resistance to several drugs have made it challenging for healthcare providers to manage and reduce the spread of this pathogen. The vast majority of isolates displayed their ability to form biofilms and carbapenemase production. However, isolates showed a high frequency of the QS genes (*abaI*/*abaR*), and virulence genes *CsgA* and *ItuA* were more abundant than the others.

6. Ethical considerations

This research was sanctioned by the research ethics committee of Hawler Medical University (Reference number: HMU.PH.EC-20212508-206).

7. Authors' contributions

QS made significant contributions to specimen collection and analysis. GA designed and supervised the research, and contributed substantially to analysis and interpretation of data. QS drafted the manuscript with assistance from GA, and GA revised it for critically important intellectual content.

8. Conflicts of interest

The authors have nothing to declare.

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