“Occurrence of bla\textsubscript{CTX-MGp1} and bla\textsubscript{CTX-MGp26} in third generation cephalosporin-resistant and carbapenem-resistant bacterial isolates from southwest region of Saudi Arabia—a preliminary study”

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A B S T R A C T

This study was intended to identify the genes responsible for ESBL- and carbapenemase-producing bacterial isolates obtained from Jizan region. A hospital-based cross-sectional study was conducted over a period of 3 months (15th November 2018–15th February 2019). Fifty non-duplicate, 3rd-generation cephalosporin and carbapenem-resistant isolates were collected from microbiology lab of a tertiary care hospital in Jizan province and were screened for ESBLs and MBLs by phenotypic methods (CDT). The positive isolates (by phenotypic method) were then scanned for the presence of \textit{bla}\textsubscript{ESBL}, and \textit{bla}\textsubscript{NDM-1} genes, respectively, by PCR.

As a result, 10% isolates showed imipenem-cephalosporin co-resistance whereas 92% (46/50) of isolates were found to be ESBL producers by CDT. The maximum occurrence was observed for \textit{bla}\textsubscript{CTX-M} (70%), followed by \textit{bla}\textsubscript{SHV} (16%) and least occurrence was noted for \textit{bla}\textsubscript{TEM} (12%). Moreover, 97% isolates (34/35) were of \textit{bla}\textsubscript{CTX-M Group1} but one isolate showed the presence of \textit{bla}\textsubscript{CTX-M Group26}. Despite the co-resistance of cephalosporin and carbapenem, 14% (7/50) were found to be MBL producer on phenotypic detection by Combination Disc Test (CDT), whereas all the isolates were found to be negative for \textit{bla}\textsubscript{NDM-1}. Hence \textit{bla}\textsubscript{CTX-M Group1} is present in quite high fraction followed by \textit{bla}\textsubscript{SHV} in the bacterial isolates of Jizan region. Moreover, the occurrence of \textit{bla}\textsubscript{CTX-M Group1} and \textit{bla}\textsubscript{CTX-M Group26} in clinical isolates from the Jizan region of Saudi Arabia has been reported for the first time.

1. Introduction

The emergence of antimicrobial resistance in microbes is a natural process but exposure to antimicrobials in healthcare, veterinary, agriculture, and the environment exaggerate it. Moreover, the onward transmission of resistance is influenced by standards of infection control, sanitation, and access to clean water, the right to use quality antimicrobials, and travel and migration. The increasing challenge of antimicrobial resistance and the subsequent absence of access to effective antimicrobials is of utmost concern worldwide. Public health faces a real threat due to improved access to antimicrobials (Laxminarayan et al., 2016). The destructive effect of this antimicrobial drug resistance is already being revealed throughout the world. Currently, the death toll due to antimicrobial resistance (in Europe and the US alone) is about 50,000 lives each year. Many more are dying in other parts of the world but knowledge of an accurate and reliable burden is still unknown (O’Neill, 2014). Understanding the scientific basis of antimicrobial resistance is essential to combat this problem.

Through the Darwinian selection process, microbes have developed various ways to evade antibiotics, like preventing entry of or expelling the drug, producing enzymes that destroy or modify the antibiotic, or modifying the antimicrobial target. Selective pressure is exerted by the antimicrobial exposure that allows microbes with inherent resistance to beta-lactams or newly acquired mutations.
or resistance genes to continue multiplying (Aminov, 2009). Antibiotic use also exerts such a selective pressure on commensal human microflora and pathogens, increasing the risk of recovery of resistant organisms from patients (Bell et al., 2014).

The term extended-spectrum beta-lactamase (ESBL) was originally applied to define TEM (refers to Temoniera) and SHV (Sulphydryl variable) beta-lactamases that can hydrolyze oxyiminocephalosporins. Moreover, the extended spectrum of activity can be described as increased hydrolysis of oxyiminocephalosporins or aztreonam by >10% than the hydrolyzing activity for benzylpenicillins. Generally, ESBLs confer resistance to 1st-, 2nd-, and 3rd-generation cephalosporins along with monobactams but remain sensitive to cephamycins and carbapenems.

A major feature of the appearance of multi-drug-resistant Gram-negative bacilli in hospitals has been the spread of extended-spectrum β-lactamases, at first in Europe and then throughout the world (Sirot, 1995). Also, the majority of beta-lactamases spread via mobile genetic elements that possess transmissible resistance factor for other antibiotic classes. This indicates that antibiotic resistance arises because of the multiplicity of selection pressure (Bush, 2018). blacTX-M is emerging at a fast pace and up to 182 blacTX-M variants have already been reported (http://www.lahey.org/studies/other/asp#table). Escherichia coli and Klebsiella pneumoniae found to be the most common species that produce ESBLs (Liu et al., 2015). Later, CTX-M-15 and CTX-M-9 were discovered, of which CTX-M-15 was the predominant type (Ensor et al., 2006). In contrast to other MBLs, NDM-1 quickly spread worldwide. Being a prominent carbapenemase on the Indian subcontinent, it also shows major outbreaks in the Balkans and Middle East (Pitout and Nordmann, 2015; Logan and Weinstein, 2017). Recently, WHO developed a global priority pathogen list of antibiotic-resistant bacteria for which there is an urgent need for new treatment. These bacteria were selected based on different criteria like all-cause mortality, healthcare and community burden, prevalence of resistance, 10-year trend of resistance, transmissibility, preventability in hospital and community settings, etc. The pathogens are stratified as “Critical”, “High”, and “Medium” tiers (http://www.who.int/medicines/news/releases/2017/bacteria-antibiotics-needed/en/). The “Critical” tier includes carbapenem-resistant Acinetobacter baumannii and Pseudomonas aeruginosa and Enterobacterial isolates that are resistant to carbapenem and 3rd-generation cephalosporin.

There is a paucity of literature regarding molecular surveys of antimicrobial resistance genes in the Kingdom of Saudi Arabia, and more specifically from the southwest region (Jizan province). Hence, the present study was designed to ascertain the genes responsible for ESBL- and carbapenemase-producing bacterial isolates obtained from Jizan region.

2. Material and methods

2.1. Ethical approval

Ethical approval to conduct this study was obtained from the Research Ethics Committee of Jazan University and the Ethical Committee of King Fahad Hospital (Abu Arish, Jizan Province). The well-being, health, safety, and confidentiality of participants was maintained throughout the study. The identity of the participants was kept anonymous in the present study.

2.2. Bacterial samples

A hospital-based cross-sectional study was conducted over a period of 3 months (15th November 2018–15th February 2019). Fifty non-duplicate, 3rd-generation cephalosporin and carbapenem-resistant isolates were collected from various clinical samples such as urine, blood, pus, endotracheal aspirate, vaginal swab, catheter tip, CSF, and stool that were received in the Microbiology Laboratory for routine culture and sensitivity, from both inpatient and outpatient departments. The isolates were cultured and identified by VITEK 2 (Biomerieux, SA, Marcy, l’Etoile, France) according to the manufacturer’s instructions. Sub-cultures of 3rd-generation cephalosporin-resistant Enterobacterial isolates, carbapenem-resistant Acinetobacter baumannii, and Pseudomonas aeruginosa, which were obtained from the laboratory unit of King Fahad Hospital (Abu Arish, Jazan, KSA), were transferred to the molecular biology laboratory of the Faculty of Public Health and Tropical Medicine for further testing.

2.3. Antibiotic susceptibility testing

The quality control strains used were K. pneumoniae ATCC700603 (as the positive control strain) and E. coli ATCC25922 (as the negative control strain). Antimicrobial susceptibility testing for all the test isolates was done by the VITEK2 system according to the manufacturer’s instructions. The results were interpreted as per Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2014) for the following antibiotics.

(i) Cephalosporin: 4th generation: Cefepime
3rd generation: Cefotaxime, Ceftazidime, Ceftriaxone
2nd generation: Cefuroxime

(ii) Monobactam: Aztreonam
(iii) Quinolone: Ciprofloxacin, Levofloxacin
(iv) Glycylcycline: Tigecycline
(v) Folate Pathway Inhibitor: Trimethoprim/Sulphamethoxazole
(vi) Aminoglycoside: Amikacin
(vii) Carbapenem: Imipenem, Meropenem

2.4. Phenotypic screening for ESBLs

Screening for ESBL producers was done by disc diffusion using ceftoxime (30 µg), cefotaxime (30 µg), ceftriaxone (30 µg), or aztreonam (10 µg) in accordance with CLSI guidelines. Combination Disc Test (CDT) was done for the detection of ESBL-producers in accordance with the recommendations of CLSI guidelines. Discs of ceftazidime (30 µg) and cefotaxime (30 µg) with or without clavulanate (Becton Dickinson, USA) were placed on a Mueller Hinton Agar plate (‘Saudi Preparaed Media Laboratory, Riyadh, Saudi Arabia’). An increase of ≥5 mm in the zone size of the antibiotic disc containing clavulanate as compared to the zone of the corresponding disc without clavulanate indicates ESBL producers.

For the isolates found positive by this approach, the gene responsible for the particular phenotype was identified by PCR (S1000™ BioRad, Thermal Cycler) with primers (synthesized by Macrogen) targeting the encoding gene.

2.5. Detection and characterization of blacTX-M

The DNA template was prepared by the boiling lysis method. A total of 4–5 bacterial colonies were dispensed in 50 µL of miliQ water and subjected to heating at 95 °C for 5 min and then sudden cooling at 4 °C for 2 min in a thermal cycler.

The isolates were then examined for blacTX-M alleles by a multiplex PCR (Woodford et al., 2006) designed to identify all genogroups of a blacTX-M. The cycling conditions that were used in the above-mentioned protocol are as follows: initial denaturation at
95 °C for 5 min; 25 cycles of 94 °C for 1 min; 63 °C for 30 sec and 72 °C for 30 sec; and a final elongation at 72 °C for 10 min. Primers used were:

- CTX-M gp1F (5′-ATA TAT GAG TAA ACT TGG-3′) CTX-M gp1R (5′-AGC TTA TTC GCC GCA TTG-3′);
- CTX-M gp2F (5′-CCA CGC TAC CCC TGT TAT-3′) CTX-M gp2R (5′-CCA GCG TCA GTA GCC GTG-3′);
- CTX-M gp9R (5′-ATT AGA AAG CGT TCA ACC-3′);
- CTX-M gp26F (5′-GCA CGA TCG TAT TCG GC-3′) CTX-M gp26R (5′-AAC CCA CGA TGT GGG TAG C-3′).

The expected amplicon for blaCTX-M-Gp1 is at 415 bp, blaCTX-M-Gp2 is at 552 bp, blaCTX-M-Gp9 is at 205 bp, blaCTX-M-Gp26 is at 666 bp and blaCTX-M-Gp26 is at 327 bp.

2.6. Detection of blaTEM and blaSHV

The isolates were examined for blaTEM and blaSHV genes according to the previously published protocol (Shahid, 2010). The cycling conditions for detecting both genes were the same: initial denaturation at 95 °C for 15 min; 35 cycles of 94 °C for 1 min; 58 °C for 2 min and 72 °C for 3 min; and a final elongation at 72 °C for 10 min. Primers used in the study were as follows:

- TEM-F (5′-KAC AAT AAC CCT GRT AAA TGC-3′) TEM-R (5′-AGT ATA TAT GAA TAA ACT TGG-3′);
- SHV-F (5′-TAT ATC GCC CTT CAT ACA AGG-3′) SHV-R (5′-GCT GCC GCC GGC ATA AGG-3′) where, Y wobble (C + T); R wobble (A + G); K wobble (G + T).

The expected amplicon for blaSHV is at 930 bp and for blaTEM is at 936 bp.

2.7. Phenotypic detection of MBLs

The isolates showing reduced susceptibility to imipenem were selected for phenotypic screening of MBLs by the CDT method (Bashir et al., 2011). An overnight culture of 0.5 McFarland turbidity standard of the test isolate was swabbed on Mueller-Hinton Agar plates. An imipenem disc with 4 μl (of 750 μg) 0.5 M EDTA (Ethylene Diammine Tetraacetic Acid, Sigma, USA) solution and without EDTA was placed on the plate. The plates were incubated at 35 °C for 24 h. An increase of >5 mm zone diameter around the disc was considered a positive result.

2.8. Detection of blaNDM-1

The blaNDM-1 gene was identified as per the previously published protocol (Peirano et al., 2011). Cycling conditions were as follows: initial denaturation at 95 °C for 5 min; 30 cycles of 95 °C for 1 min; 52 °C for 1 min and 72 °C for 60 sec; and a final elongation at 72 °C for 5 min with the primers NDM-F (5′-CAC CGG AGC TTG TGC-3′) and NDM-R (5′-TGC CGA AGC TGA GCA-3′). The expected amplicon for blaNDM-1 lies at 800 bp.

2.9. Analysis of amplified PCR products

PCR products were resolved on 2.0% agarose gels, stained with ethidium bromide and photographed with the Gel Doc XR + Gel Documentation system (BioRad) (Alshammary and Khan, 2021).

2.10. Statistical analysis

Statistical analysis was done as per the studies of Khan et al., (2019).

3. Results

In the present study, 64% (32) of patients were male and 36% (18) were female. The range of the age of patients who contributed the maximum number of isolates (20%) was between 21 and 30 years; least isolates (2%) were procured from the age group 11–20 years and also from the age group 61–70 years. The details are shown in Table 1.

Our study isolates were characterized as Escherichia coli (25), Klebsiella pneumoniae (20), Acinetobacter baumannii (2), Pseudomonas aeruginosa (2), and Enterobacter cloacae (1). The maximum number of isolates was obtained from pus (34%), followed by urine (24%) and blood (20%). Details are shown in Table 2.

In the present study, the maximum number of isolates was obtained from the surgery ward (22%) followed by the intensive care unit (16%) and the NICU (14%), whereas the least number of isolates was obtained from the burn ward (4%); this indicates the implementation of proper preventive measures regarding antibiotic usage in that particular ward (Fig. 1).

The bacterial isolates in the present study showed high resistance to extended-spectrum cephalosporins, namely, cefepime (98%), cefazidime (96%), and cefotaxime (90%), whereas resistance to ceftriaxone (82%) and cefuroxime (72%) was noted as being slightly lower. Only 42% of isolates showed resistance to aztreonam. Additionally, a quite lower proportion of isolates demonstrated resistance against carbapenems [imipenem (10%) and meropenem (12%)]. Moreover, all the study isolates were found to be sensitive to ticarcilin. The antibiotic resistant rate and pattern are demonstrated in Fig. 2.

Up on phenotypic detection of ESBLs, we found that 92% (46/50) of isolates showed positivity by combination disc test (CDT) using ceftazidime (30 μg) and cefotaxime (30 μg) with and without clavulanate. The isolates were then subjected to genotypic detection for various bla genes to determine the exact molecular mechanism responsible for antibiotic resistance. Maximum occurrence was noted for blaCTX-M (70%), followed by blaSHV (16%) and blaTEM (12%). Among CTX-M producers, 97% of isolates (34/35) were found to belong to blaCTX-MGroup1 (415 bp), though blaCTX-M genusgroup26 (327 bp) was also noted in one isolate. The isolate that showed the presence of CTX-M group 26 was obtained from the urine sample of an 88-year-old male patient from the urology ward. The increased level of blaCTX-M in bacterial isolates could be attributed to inappropriate and excessive use of antibiotics in empirical treatment.

Despite the co-resistance of cephalosporin and carbapenem, only 14% (7/50) of isolates were found to be MBL-producer on phenotypic detection by the Combination Disc Test (CDT). All the isolates were found to be negative for blaNDM-1, on genotypic detection by PCR. Perhaps some other gene is responsible for carbapenem resistance in our study isolates. Luckily, we did not notice a combination of various bla genes in these isolates, indicating that the situation is still not very complex in the Jizan hospital.

Table 1

| Age  | Number of patients |
|------|--------------------|
| <1 year | 7 (14%) |
| 1–10 years | 6 (12%) |
| 11–20 years | 1 (2%) |
| 21–30 years | 10 (20%) |
| 31–40 years | 7 (14%) |
| 41–50 years | 6 (12%) |
| 51–60 years | 6 (12%) |
| 61–70 years | 1 (2%) |
| 71–80 years | 4 (8%) |
| 81–90 years | 2 (4%) |
In the bacterial population, various resistance mechanisms have recently emerged, including alteration of target (a DNA gyrase), increased efflux (export of a drug out of the microorganism), fluoroquinolone inactivation (by an aminoglycoside N-acetyltransferase), and protection of the target by DNA-binding proteins (known as Qnr) (Redgrave et al., 2014). Antimicrobials are among the most commonly prescribed drugs used in human medicine but it has been noticed that up to 50% of all prescribed antimicrobials are unnecessary (Center for Disease Control and Prevention, 2013) (http://www.cdc.gov/drugresistance/pdf/ar-threats-2013–508.pdf).

Table 2
Bacterial isolates and the sample of origin.

| Sample                          | Escherichia coli | Klebsiella pneumoniae | Acinetobacter baumanii | Pseudomonas aeruginosa | Enterobacter cloacae |
|--------------------------------|------------------|-----------------------|------------------------|------------------------|---------------------|
| Pus (n = 17)                   | 9                | 7                     | –                      | 1                      | –                   |
| Urine (n = 12)                 | 9                | 2                     | 1                      | –                      | –                   |
| Blood (n = 10)                 | 4                | 6                     | –                      | –                      | –                   |
| Endotracheal Aspirate (n = 6)  | –                | 4                     | 1                      | –                      | 1                   |
| Catheter Tip (n = 1)           | –                | –                     | –                      | –                      | –                   |
| Vaginal Swab (n = 2)           | 2                | –                     | –                      | –                      | –                   |
| CSF (n = 1)                    | –                | 1                     | –                      | –                      | –                   |
| Stool (n = 1)                  | 1                | –                     | –                      | –                      | –                   |
| Total                          | 25               | 20                    | 2                      | 2                      | 1                   |

Fig. 1. Distribution of bacterial isolates in various wards.

Fig. 2. Antibiotic resistant rate in bacterial isolate.

4. Discussion

Antimicrobial use exerts selective pressure on commensal human microflora along with pathogens and hence, increases the risk of the recovery of resistant organisms from patients (Bell et al., 2014). The use of antibiotics in clinical care is responsible for in vivo development of resistance (Elliott and Brink, 2006). It has been reported by WHO (2012) that antibiotic resistance emerged (at least a fragmentary proportion) as a result of selective pressure exerted by antibiotic use in veterinary medicine and agriculture (WHO, 2012). Evidence supporting the transmission of ESBLs and AmpC genes on E. coli plasmids most likely through the food chain has also been reported (Kluytmans et al., 2013).

It has been noted that the concentration of antibiotic prescribing might be highest in inpatient settings, with 30–40% of patients...
on antibiotics in European hospitals (European Centre for Disease Prevention and Control, 2013) (http://ecdc.europa.eu/en/health-topics/healthcare-associated_infections/point-prevalence-survey/Documents/healthcare-associated-infections_antimicrobial_use-PPS-summary.pdf), whereas, the overall quantity of antimicrobial prescription is highest in the community (ECDCP. Surveillance of antimicrobial consumption in Europe, 2012) (http://ecdc.europa.eu/en/publications/Publications/antimicrobial-consumption-europe-esac-net-2012.pdf). Moreover, public health factors like the role of migration and tourism, sanitation, and population densities also affect the prevalence of antimicrobial resistance (Turnidge and Christiansen, 2005).

Meanwhile, the transmission of drug-resistant organisms in health care institutions depends on the duration of the patient stay and the contamination of health care workers’ hands and fomites (Chamchod and Ruan, 2012). Another factor that contributes to the continuing transmission of resistant organisms in healthcare settings is the inability to rapidly identify resistant organisms with appropriate and efficient diagnostic tests (Holmes et al., 2016). The recent spread of carbapenem-resistant genes (NDM, KPC, and OXA-48) is of greatest concern (Johnson and Woodford, 2013).

In a study from Saudi Arabia, Bindayna et al. (2010) identified blaCTX-M as the predominant genotype among ESBL producers. Moreover, Yasir et al. (2018) reported the occurrence of blaCTX-M isolates belonging primarily to the group blaCTX-M-1 (74.6%), followed by blaCTX-M-9 (20.4%), and also reported the occurrence of blaoxy in 5.2% of isolates only and blaTEM in 83.9% of isolates. They have also reported the presence of bla genes combination with most common one was being blaCTX-M + blaoxy (79.1%). In our study isolates, blaoxy was noted in 16% of isolates only and blaTEM was found to be present in 12% of isolates. These findings are somewhat contrary to those observed by Yasir et al. (2018). The difference may be attributed to varied antibiotic prescription methods in the western and southern regions of Saudi Arabia.

Tawfik et al. (2011) conducted a study in the AlQassim region of Saudi Arabia and reported a 25.6% prevalence of ESBLs in Klebsiella pneumoniae isolates. They observed that all the ESBL-positive clinical isolates were sensitive to imipenem and tigecycline. blaCTX-M was noted in 36.4% of isolates (CTX-M-15 in 34.5% and CTX-M-14 in 1.8%), blaoxy in 89.1%, and blaTEM in 70.9%. Recently, Alqasim et al. (2018) reported a 93.94% prevalence of CTX-M beta-lactamase genes in E. coli from the Riyadh region. All the reported isolates belonged to the CTX-M group 1 and the researchers did not find blaoxy in their study isolates. Moreover, Al-Agamy et al. (2018) reported the isolation of carbapenem-resistant E. coli including NDM-1 and OXA-4 from a hospital in Riyadh. Recently, a carbapenem-resistant isolate was detected in municipal wastewater in Saudi Arabia (Mantilla-Calderon et al., 2016). Moreover, AlOtaibni et al. (2017) reported the presence of carbapenem-resistant Enterobacteriaceae from a hospital in Saudi Arabia. In recent times, blaoxy has been identified in clinical isolates from Jizan province (Basode et al., 2018). In contrast to these reports, we did not observe the presence of blaoxy in our study isolates despite the presence of carbapenem resistance; it may be possible that other class B beta-lactamase genes were present in those isolates and were responsible for the noted resistance against imipenem and meropenem.

Previously reported studies from Saudi Arabia displayed a significant rise in resistance rates to various antimicrobial agents during the past years. The resistance rate for individual cephalosporins (such as cefuroxime, cefotaxime, cefazidime, and cefepime) was considerably high as compared to beta-lactam and beta-lactamase inhibitor combination (amoxicillin-clavulanic acid and piperacillin-tazobacan) (Uz Zaman et al., 2018; Elabd et al., 2015; Al-Agamy et al., 2014, 2016; Al-Zahrani and Alasiri, 2018; Alyamani et al., 2017; Khan and Faiz, 2016; Mashwal et al., 2017). Moreover, it has been reported that during the initial study period of year 2000, ESBL prevalence was relatively low (4.8%), but increased dramatically to 72% by 2020. The PCR analysis of various beta-lactamases traits demonstrates that the prevalence of blashv, blatem and blaCTX-M among Enterobacterial isolates increased from between 2.6 and 8.5 to 84.1–97.3% (Nasser et al., 2020).

This is the first report indicating the occurrence of blaCTX-M Group 1 and blaCTX-M Group 26 in clinical isolates from the southwest region of Saudi Arabia. The amplified products were sent for DNA sequencing so that the CTX-M could be identified exactly.

The present study has a few limitations, including the screening of carbapenem isolates for the presence of other class B beta-lactamase genes, for example, blavim, blaimp, blakpc, etc. Additionally, the study should be performed with an increased sample size and over a large span of time for purposes of identifying the actual pattern of resistance genes in the southwest region of the kingdom and so that proper measures can be taken to limit the spread of antibiotic resistance isolates.

Moreover, raising awareness of the fundamentals of antibiotic use and the issue of antibiotic resistance in public is necessary. Above all, surveillance should be improved to preserve the effectiveness of antimicrobials and delay the resistance issue in humans. However, our knowledge and understanding of antibiotic resistance are still incomplete. There is no single solution to combat this problem and there is a need for several intersecting and synergistic approaches coordinated at the national and international levels.

Transparency declaration

Nothing to declare.

Ethics approval

Ethical approval was obtained from the Research Ethics Committee of Jazan University and the Ethical Committee of King Fahad Hospital (Abu Arish, Jazan Province).

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author contribution statement

FS conceived and designed research. FS performed experiments and SNQ collected sample from hospital. SNQ and KYG analyzed data and helped in writing manuscript. FS wrote the manuscript. All authors read and approved the manuscript for publication.
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