ORIGINAL ARTICLE

In vitro efficacy of colistin against multidrug-resistant *Pseudomonas aeruginosa* in burn patient by minimum inhibitory concentration with broth dilution

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ABSTRACT

Background: *Pseudomonas aeruginosa* strains cause 86% of sepsis mortality in burn victims. Therefore, to combat the multi-drug resistance *in Pseudomonas aeruginosa*, colistin is the new drug, and it has recently been introduced. To analyze the in vitro efficacy of colistin against multidrug-resistant *Pseudomonas aeruginosa* in burn patients by determining the minimum inhibitory concentration with broth dilution.

Material And Methods: The cross-sectional study was performed from March 2021 to February 2022 in the Burn Centre at Nishter Hospital in Multan, Pakistan. 300 burn patients (≥20% burn) were selected, and their pus samples were collected and processed in the microbiology laboratory. The Kirby-Bauer disc diffusion method was applied to check the antibiotic susceptibility against isolated strains. The colistin sensitivity against multi-drug resistant (MDR) strains was estimated by employing the broth dilution method at two-fold serial dilutions from 0.5 µg/mL to 0.003 µg/mL.

Results: 124 (55.3%) strains were identified as *Pseudomonas aeruginosa*, while the remaining strains were identified as *Escherichia coli* (13.0%), *Streptococci* (13.0%), *Klebsiella pneumoniae* (8.9%), *Staphylococci* (6.6%), and *Candida albicans* (2.2%). All isolated strains of *Pseudomonas aeruginosa* showed resistance against antibiotics: aztreonam (60.4%), cefepime (72.5%), ceftazidime (71.7%), ciprofloxacin (62.0%), imipenem (33.0%), levofloxacin (58.0%), meropenem (19.3%), piperacillin/tazobactam (66.1%), and tobramycin (63.7%). The significantly calculated MIC value of colistin against MDR strains was 0.35-0.5 mg/L (CLSI and EUCAST recommended value = ≤2 mg/L).

Conclusion: Colistin can be a good option to treat nosocomial infections of MDR *Pseudomonas aeruginosa* in burn patients.

Keywords: Burn patients, Colistin, Multi-drug resistance, Minimum inhibitory concentration

BACKGROUND

Skin is the largest organ of the human body and regulates homeostasis and acts as a protective barrier against infections.[1] Damage to the skin causes loss of the skin barrier and subsequent pathogenic inflammatory activation.[2] The high mortality rate from skin burns is unacceptable and underreported.[3] Direct or indirect contact with chemicals, fire, and electric current can cause mild or severe skin burn injuries.[4] According to the World Health Organization (WHO), burn injuries cause 180,000 deaths globally each year. Pakistan's 6.5% burn death rate is a result of inadequate burn patient care and inadequate infrastructure.[5] Patients with burns are also more susceptible to bacterial and fungal infections during their course of treatment due to an infirm immune system.[6, 7]

The opportunistic pathogenicity of *Pseudomonas aeruginosa* (*P. aeruginosa*) is widely recognized in patients with impaired immune systems.[8] Nosocomial infections (NIs) are frequently caused by *P. aeruginosa* in burn patients.[9] Bloodstream infections (BSIs), surgical site infections (SSIs), and urinary tract infections (UTIs) are examples of these NIs.[10,12] Approximately 10-11% of NIs are caused by *P. aeruginosa*.[13] In 2017, the WHO identified *P. aeruginosa* as a pathogen that needed to be studied more closely to develop new antibiotics.[14]

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Antibiotic resistance is one of the major reasons for the high mortality rate in burn patients from *P. aeruginosa* NIs.[15] 86% of sepsis deaths in burn patients are caused by multi-drug resistant (MDR) strains of *P. aeruginosa*.[16] It was discovered that carbapenems were the most successful antibiotics against *P. aeruginosa* NIs.[17] However, *P. aeruginosa* strains that are resistant to β-lactams, carbapenems, cephalosporins, aminoglycosides, and quinolones have been reported in previous years.[18]

Colistin and polymyxin B (previously believed to be toxic for clinical use) are currently being brought back to be used as "last option" antibiotics against Gram-negative bacteria.[19] In 1947, Koyama discovered colistin as a byproduct of the Gram-positive soil bacteria *Paenibacillus polymyxa* subsp. *Colistinus* in Japan.[20] Colistin began to be used in both humans and animals in 1952. But between the 1970s and 1980s, its utilization in medicine nearly disappeared. However, recently, to treat infections caused by the MDR *P. aeruginosa*, colistin has started to be used in humans.[20] The WHO recommended colistin as critically important human medicine in 2018.[21] Therefore, the present study was conducted to determine the frequency of MDR *P. aeruginosa* in burn patients and the minimum inhibitory concentration (MIC) value of colistin using the broth dilution method against MDR strains

MATERIAL AND METHODS

The cross-sectional investigation was carried out at the Microbiology Laboratory, Department of Pathology, Nishter Medical University, in association with the Pak Italian Burn Unit in Multan. The convenient non-probability sampling technique was used. A sample size of 124 MDR *P. aeruginosa* isolates was calculated by taking a 95% confidence level, 5% absolute precision, and an expected prevalence of 22.7% of *P. aeruginosa* in burn patients.[22] The burn patients (male and female), representing any type of burn and ranging from 15 to 60 years of age, were included in this study. Patients with less than 20% burn, MLC cases as per hospital records, and patients taking antibiotics were excluded.

For this study, a total of 300 patients with severe burns were chosen. Every patient was admitted to the Nishter Hospital Burn Centre. The basic information, medical history, and history of infections were gathered using a specified questionnaire. Furthermore, each patient provided written approval for the use of their samples.

The aspirators or drainage tubes were used to gather pus samples in sterile containers from each patient. The sterile cotton swabs were used to help obtain pus samples from the burn wounds of some patients who did not have any drainage. Afterward, every pus sample was appropriately labeled and sent to the microbiology lab for further investigation (Figure-I).

The selected antibiotics for this study are mentioned in Table-I. The sterile and freshly prepared Muller-Hinton agar was dispensed into the sterile petri plates, and the poured plates were kept at room temperature until solidification. The in vitro antibiotic susceptibility of each strain was ascertained using the Kirby-Bauer disc diffusion method. Using aseptic methods, bacterial suspensions (0.5 MacFarland) were swabbed onto each agar plate. The sterile syringes were used to impregnate antibiotic discs on agar plates at equal distances. Following incubation, zones of inhibition (ZOIs) were recorded and interpreted in accordance with Clinical and Laboratory Standard Institute (CLSI) recommendations of 2022. For MIC determination, a stock solution of colistin (Sigma-Aldrich) was prepared in a clean test tube. For this, 10 mg was added to 100 mL of sterile deionized water and thoroughly mixed. From this tube, 1 mL was transferred to another test tube containing 100 mL of sterile deionized water. This test tube was labelled as A. 11 clean test tubes were taken, and labelled from 1 to 11. 0.5 mL of freshly prepared nutrient broth was added to test tubes 2 to 11. 0.5 mL of antibiotic solution (test tube A) was added to test tubes 1 and 2. Two-fold serial dilutions of the antibiotic solution were prepared. From test tube 2, 0.5 mL was transferred to test tube 3 and mixed well. The same procedure was continued up to test tube 9. From tube 9, 0.5 mL was discarded to equal the volume (0.5 mL) in each tube. 0.5 mL of *P. aeruginosa* overnight-grown culture suspension (turbidity equal to 0.5 MacFarland standard) was added in each test tube except test tube 11. Finally, each test tube received a total volume of 1 mL, while consecutive test tubes received one and half of the original concentration of antibiotic. The test tubes 01, 10, and 11 were considered controls. Test tube 01 was an antibiotic control tube (it received 0.5 mL of bacterial suspension and 0.5 mL of antibiotic). The test tube was labelled C_1. Test tube 10 was a growth control

tube (it received 0.5 mL of bacterial suspension and 0.5 mL of nutrient broth). The test tube was labelled C_2. Test tube 11 was a sterility control tube (it received only 0.5 mL of nutrient broth). The test tube was labelled C_3. All test tubes were kept in an incubator at 37 °C for 22-24 hours, and the next day, visible turbidity in the test tubes was observed (Figure-II). To calculate the MIC value, the following formula was used:

MIC value=(lowest dilution inhibits growth + consecutive highest dilution allows growth) / 2

Excel spreadsheet version 22 and SPSS software version 25 were used to enter and analyze data, respectively. We noted the percentages or frequencies of all variables (age, gender, burn types, and isolated strains) accordingly.

RESULTS

The age distribution results showed that 20 (13.0%), 43 (28.7%), 56 (37.3%), 24 (16.0%), and 7 (4.6%) burn patients belonged to the age range of <20 years, 20-30 years, 31-40 years, 41-50 years, and >50 years old, respectively. The occurrence of burning was observed more in males (56%) than females (43%). In the present study, 23 (15.3%) patients of acid burn, 18 (12.0%) patients of accident burn, 16 (10.6%) patients of electrical burn, 40 patients (26.6%) of flame burn, 21 patients (14.0%) of liquid burn, 23 patients (15.3%) of scald burn, and 8 (5.3%) patients of suicide burn cases were observed. After biochemical testing, 124 (55.3%) bacterial strains were characterized as *P. aeruginosa*. Similarly, 100 strains out of the remaining 150 were identified as *Escherichia coli* (13.3%), *Streptococci* spp. (13.3%), *Klebsiella pneumoniae* (8.9%), *Staphylococcus* spp. (6.6%), and *Candida albicans* (2.2%).

The susceptibility of 124 strains of *P. aeruginosa* was further tested against aztreonam, cefepime, ceftazidime, ciprofloxacin, levofloxacin, piperacillin/tazobactam, and tobramycin. The measured ZOIs were compared with the CLSI guidelines of 2021, and it was observed that all 124 strains of *P. aeruginosa* were MDR. Out of a total of 124, 75 (60.4%), 90 (72.5%), 89 (71.7%), 77 (62.0%), 41 (33.0%), 72 (58.0%), 24 (19.3%), 82 (66.1%), and 79 (63.7%) *P. aeruginosa* strains were resistant to aztreonam, cefepime, ceftazidime, ciprofloxacin, imipenem, levofloxacin, meropenem, piperacillin/tazobactam, and tobramycin, respectively.

The broth dilution method was employed to calculate the MIC value of colistin against MDR strains of *P. aeruginosa*. Two-fold serial dilutions from 0.5 µg/mL to 0.07 µg/mL of colistin were prepared in test tubes. After the subsequent incubation period, all broth test tubes were clearly observed for the presence or absence of turbidity. The optical density (OD_{600}) values of each test tube were measured using a spectrophotometer (Table-II). According to the visual observation and OD_{600} values, test tube no. 2 showed no turbidity with an OD_{600} value of 0.12 as compared to other dilutions of colistin. After repetitive experiments, an average MIC value of 0.375-0.5 µg/mL (0.35-0.5 mg/L) was estimated for MDR strains of *P. aeruginosa* (Figure-III). This MIC value falls within the colistin MIC breakpoints (Table-III) of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and Clinical Laboratory and Standards Institute (CLSI) against MDR strains.

Table-I: Antibiotic discs used for antibacterial susceptibility testing.

Sr. No.	Name of Antibiotic	Disc content
1.	Aztreonam (ATM)	30 µg
2.	Cefepime (FEP)	10 µg
3.	Ceftazidime (CAZ)	30 µg
4.	Ciprofloxacin (CIP)	5 µg
5.	Levofloxacin (LEV)	5 µg
6.	Piperacillin/Tazobactam (TZP)	(100/10 µg)
7.	Tobramycin (TOB)	10 µg
8.	Meropenem (MEM)	5 µg

Table-II: OD_{600} values of different colistin concentrations and controls.

Test tube number	Colistin concentration (µg/ml)	Optical density (OD) value
C_1	0.5	0.1
C_2	0	1.4
C_3	0	0.03
2	0.5	0.12
3	0.25	0.23
4	0.125	0.34
5	0.06	0.48
6	0.03	0.57
7	0.015	0.69
8	0.007	0.85
9	0.003	0.97

Table-III: Colistin MIC breakpoints.

Bacteria	EUCAST Breakpoints (mg/L)		CLSI Breakpoints (mg/L)	
	S≤	R>	I≤	R>
Enterobacteriaceae	2	2	2	4
Pseudomonas	2	2	2	4
Acinetobacter	2	2	2	4

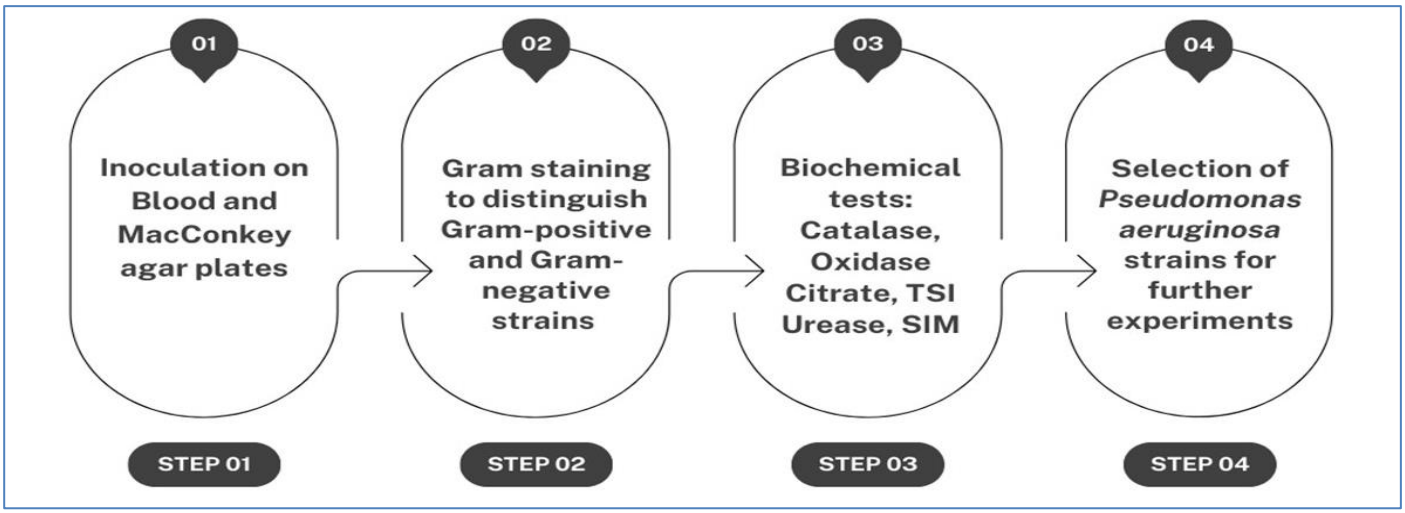

Figure-I: Flowchart shows the processing of the collected pus samples.

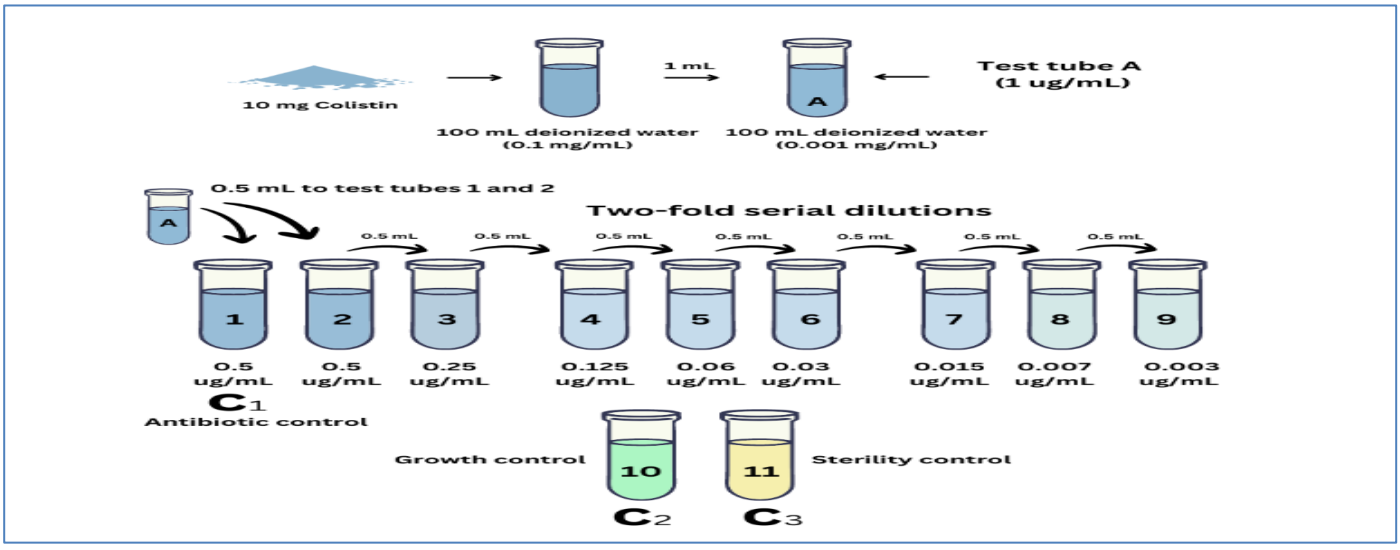

Figure-II: Graphical representation of the MIC determination by broth dilution method.

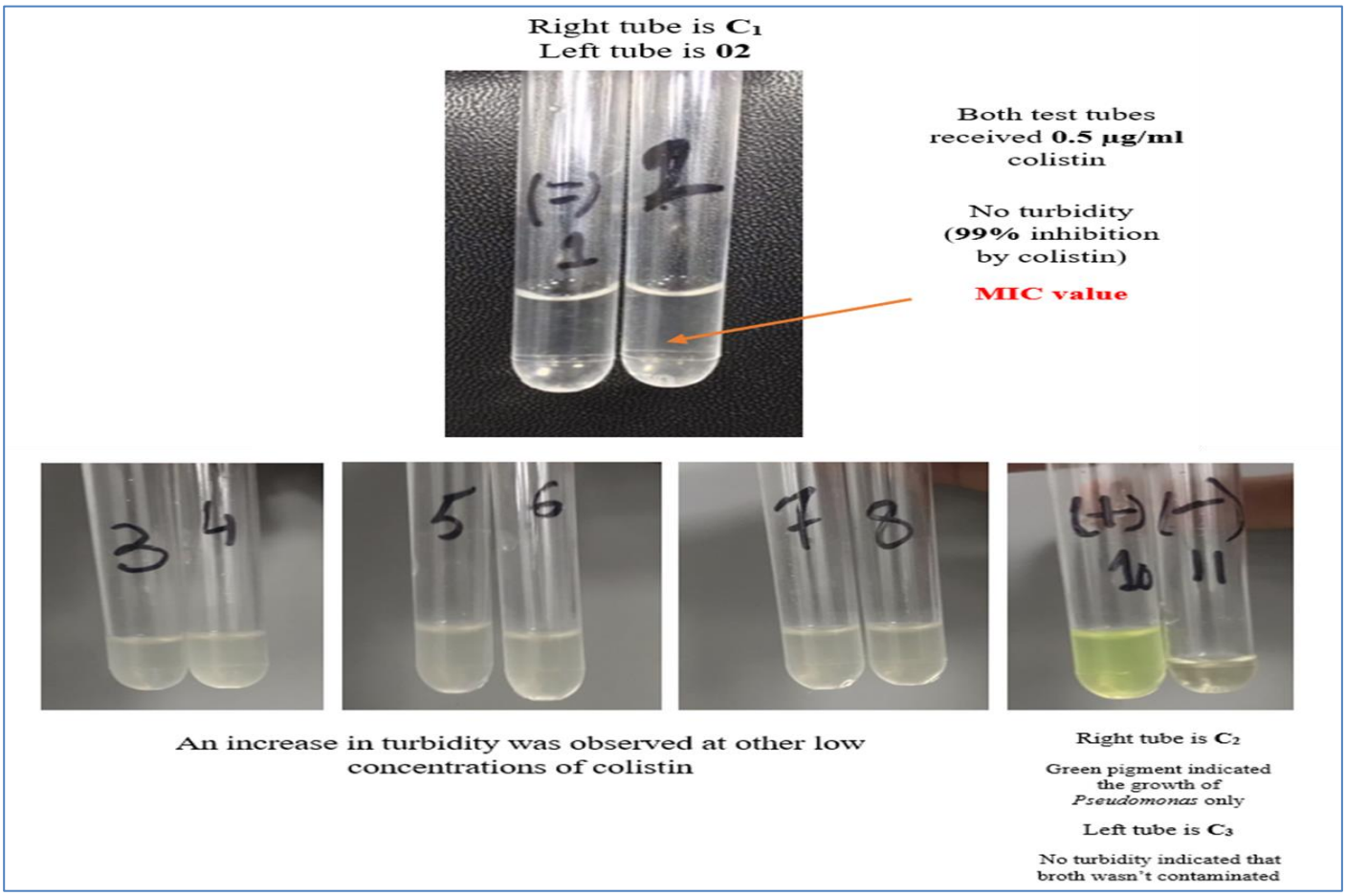

Figure-III: Results of the broth dilution method to determine the colistin MIC value against MDR *P. aeruginosa*.

DISCUSSION

A burn percentage of >20 was observed in all age groups and both genders. It was interpreted from these results that the burn percentage among study subjects didn't depend on gender or age, while it may depend on burn types and total body surface area (TBSA). Although findings on the age factor revealed significant differences in *P. aeruginosa* infections across the groups, patients between the ages of 16 and 30, 31, and 45 were more likely to become infected with *P. aeruginosa* infections.[23] In the present study, flame, scald, acid, and liquid burn types were more prevalent among study subjects, which is comparably similar to the results of a previous study.[24] Another similar study reported that the majority of burn patients belonged to the age group of 15-60 years, accounting for 55.2%. The primary factor, which accounted for 39% of the cases, was scald burn, followed by 33.6% (flame burn), 26.6% (electrical burn), and 0.8% (chemical burn).[25]

In the present study, 124 (55.3%) pus samples presented the growth of *P. aeruginosa* on the agar plates. A previous study reported the isolation of 118 *P. aeruginosa* strains from burn patients. Similar to this, another study reported the isolation of 45 *P. aeruginosa* strains from 101 burn victims.[9,26] A recent study observed a 53.3% prevalence of *P. aeruginosa* in 2nd and 3rd degree burn patients.[27] According to the results of another recent study, the most frequently isolated MDR strain from burn patients was *P. aeruginosa*, accounting for 38%.[28] The efflux pumps, modifying enzymes, horizontal gene transfer among bacterial species, and very stable biofilm formation are responsible for the multi-drug resistance in *P. aeruginosa* clinical strains.[29] In the present study, 0.35-0.5 mg/L was estimated as the MIC value against MDR *P. aeruginosa*. According to a previous study, MIC values ≤ 2 mg/L for colistin were considered significant, while MIC values ≥ 2 mg/L were considered non-significant.[30] Another previous study t reported colistin MIC values from 0.25-2 mg/L against 12 strains of *P. aeruginosa*.[31] Another relevant study reported 99% colistin sensitivity in comparison with the antibiotics tazobactam (62%), amikacin (65%),

cefepime (65%), imipenem (62.50%), and meropenem (54%), against all clinical strains of *P. aeruginosa*.[32] Lipopolysaccharides are the main target of the colistin antibiotic in Gram-negative bacteria. The positive-charge molecule colistin has great affinity for negative-charge lipids in bacteria. The lipopolysaccharide molecules in the outer membrane cause cations to be dislodged by electrostatic interactions, rupturing the membrane, releasing lipopolysaccharide molecules into the environment, and ultimately causing cell death.[33, 34]

CONCLUSION

The present study checked the colistin sensitivity patterns among MDR strains of *P. aeruginosa*. 300 burn patients were selected for this study who were admitted to the Burn Centre at Nishter Hospital Multan, Pakistan. The pus samples were collected and aseptically transferred to the laboratory. The bacterial strains isolated from samples were identified as MDR, and a very significant MIC value of colistin was calculated by using the broth dilution method against MDR strains. In conclusion, colistin can be utilized as an effective drug against *P. aeruginosa*.

CONFLICT OF INTEREST

None

GRANT SUPPORT & FINANCIAL DISCLOSURE

Declared none

AUTHOR CONTRIBUTION

Blossom Neelam: Conception, manuscript writing, data collection, accountable for all aspects of the work

Sumera Malik: Data collection, study design, interpretation of the work, accountable for all aspects of the work

Syed Muhammad Abbas Naqvi: Study design, proofreads, accountable for all aspects of the work

Mahnoor Haidar Khan: Critical review, revisions, accountable for all aspects of the work

Mehvish Javeed: Revisions, proofreads, accountable for all aspects of the work

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