Research Article

Antimicrobial Resistance and Quorum Sensing Genes Detection among the Biofilm Forming *Staphylococcus aureus* Isolated from Admitted Patients of Dhaka Medical College Hospital, Dhaka, Bangladesh

Noor-E-Jannat Tania[1*], S. M. Shamsuzzaman[2], Aminul Islam[3]

[1]Lecturer, Department of Microbiology, Dhaka Medical College, Dhaka, Bangladesh

[2]Professor and Head of the Department, Department of Microbiology, Dhaka Medical College, Dhaka, Bangladesh

[3]Honorary Medical Officer, Department of Medicine, Dhaka Medical College Hospital, Dhaka, Bangladesh

***Corresponding author:** Noor-E-Jannat Tania, Lecturer, Department of Microbiology, Dhaka Medical College, Dhaka, Bangladesh, E-mail: noorejannat87@gmail.com

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Abstract

Staphylococcus aureus an infamous human pathogen, is a major cause of the community as well as healthcare associated infections. *Staphylococcus aureus* has the tendency to form biofilm. Biofilm formation usually increases antimicrobial resistance capacity which shows considerable challenges to successful eradication of infections. The aim of this study was to detect the biofilm forming *Staphylococcus aureus* phenotypically and to determine its association with drug resistance and also detect the prevalence of different types of quorum sensing genes among them.

Out of 275 clinical samples, 75.64% were culture positive among which 21.63%*Staphylococcus aureus* were isolated by culture and biochemical tests. Biofilm producing *Staphylococcus aureus* were isolated by tissue culture plate method and tube method. Antimicrobial susceptibility tests were performed by the standard disc diffusion technique. PCR was done to detect agr genes and sequencing of agr-I gene was done.

Tissue culture plate method was found superior to tube method for biofilm detection. Tissue culture plate method detected 71.11% and tube method detected 62.22% biofilm producers.

Biofilm-producing *Staphylococcus aureus* showed higher resistance to oxacillin 53.13%, cefoxitin 46.88% and ampicillin 46.88% than non biofilm-producers.

Among biofilm-producing *Staphylococcus aureus*, agr-I gene was predominant (46.88%) than other quorum sensing genes.

The study provided insight into the higher proportion of antibiotic resistance among non-biofilm-producing *Staphylococcus aureus* than biofilm-producers. So, antibiotic resistance is not significantly associated with biofilm-production in *Staphylococcus aureus*.

Keywords: Antimicrobial resistance; Biofilm; PCR; Quorum sensing; *Staphylococcus aureus*

1. Introduction

Staph. aureus (*Staphylococcus aureus*) is a leading cause of nosocomial infections worldwide and causative agent of a wide range of diseases. Many of these diseases, including endocarditis, osteomyelitis, and foreign body associated infections, appear to be caused by biofilm-forming *Staph. aureus* [1,2]. According to some study reports, over 65% of hospital-acquired infections occur by the infecting organisms that have the ability to biofilm production [3].

The biofilm formation is accompanied by the production of extracellular polymer and adhesion matrix and leads to fundamental changes in the bacterial growth and gene expression [4]. There are various definitions for biofilm but all of them consider three major components for it: microbes, slime exopolysaccharide and surface, removing any of them can stop producing biofilm [5].

Susceptibility to antibiotics in bacteria that are protected by biofilm is reduced because antibiotics are prevented to reach the bacteria surrounded by biofilm. Furthermore biofilm keeps bacteria out of reach of host immune defense mechanism and often resulting in persistent infections that are difficult-to-treat and life-threatening due to emergence of multidrug resistance strains and also occurrence of isolates that are able to form strong biofilms [6, 7, 8].

A large number of studies have shown that bacterial quorum sensing (QS) signaling plays important roles in biofilm production [9, 10]. The QS system of the staphylococci is called the accessory gene regulator or “agr” system. QS via agr increases production of virulence factors, which include various enzymes and toxins. Moreover, agr dysfunction is correlated with persistent *Staph. aureus* bacteremia [11].

There has been continued interest in the agr for the development of anti-staphylococcal drugs [12].Combining use of antibiotic with an anti-QS agent is the most effective clinical strategy for the treatment of bacterial diseases at present [13, 14]. In this study, we investigate the prevalence of agr groups in *Staph. aureus* isolates obtained from pus, wound swab and blood samples.

This study seeks to determine drug resistance pattern among the biofilm forming *Staph. aureus* isolated from the patients of Dhaka Medical College Hospital with prevalence of quorum sensing genes among them by PCR assays.

2. Materials and Methods

This Cross sectional study was conducted from 1 January 2019 to 31 December 2019 in the Microbiology laboratory of Dhaka Medical College Hospital, Dhaka, Bangladesh.

2.1. Samples collection and identification

Samples were collected from pus, wound swab and blood of clinically suspected infected patients of inpatient departments of Dhaka Medical College Hospital or were received in the microbiology department for culture and sensitivity after taking informed written consent irrespective of age, sex and antibiotic intake. Patient who did not give consent were excluded from this study. *Staph. aureus* were identified by Gram staining, catalase test, coagulase test (slide and tube method), colony morphology, hemolytic property, pigment production and mannitol fermentation test in mannitol salt agar media as per standard procedures [15].

2.2. Biofilm experiments

Biofilm formation by these isolates was detected by two in vitro methods: tissue culture plate method and tube method [16, 17].

2.2.1. Tissue culture plate method

The organisms were inoculated in 10 mL of trypticase soya broth (TSB) with 1% glucose at 37°C or 24hours. The cultures were then diluted 1:100 with fresh medium and individual wells of sterile 96 polystyrene tissue culture plates were filled with 200 µl of the diluted cultures. Negative control wells contained inoculated sterile broth. The plates were incubated for 24 hours at 37°C. The wells were gently washed four times with 0.2ml of phosphate buffered saline (PBS) (PH 7.2), fixed by sodium acetate (2%) and stained by 0.1% crystal violet. Excess stain was removed by deionized water, and the plate was kept for drying. The optical density at 570nm (OD570) was determined using a micro-ELISA auto-reader assay. The experiment was performed in triplicate and repeated three times.

2.2.2. Calculation of OD values [18]

The average OD values were calculated for all tested strains and negative controls, since all tests were performed in triplicate and repeated three times. Second, the cut off value (ODc) was established. It was defined as three standards (SD) above the mean OD of the control: ODc = average OD of negative controls + (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). ODc value was calculated for each microtiter plate separately. If a negative value obtained, it should be present as zero, while any positive value indicates biofilm.

2.2.3. Interpretation of biofilm production by TCP method

Average OD value	**Biofilm production**
OD ≤ ODc	Non biofilm producer
ODc < OD ≤ 2× ODc	Weak biofilm producer
2 × ODc < OD ≤ 4 × ODc	Moderate biofilm producer
4 × ODc < OD	Strong biofilm producer

2.2.4. Tube method

TSB with 1% glucose (10ml) was inoculated with loopful of microorganism from overnight culture plates and incubated for 24 hours at 37°C. The tubes were decanted and washed with PBS (pH 7.2) and dried. Dried tubes were stained with crystal violet (0.1%). Excess stain was removed and tubes were washed with deionized water. Tubes were than dried in inverted position and observed for biofilm formation. Biofilm formation was considered positive when a visible film lined the wall and bottom of the tube. Experiments were performed in triplicate and repeated three times. The adherence property of biofilm producers was graded as strong, moderate and weak and non-biofilm producer.

2.3. Antimicrobial susceptibility test

Antimicrobial susceptibility tests of the clinical isolates against different antimicrobials were performed in Muller-Hinton agar (MHA) using the standard disk diffusion technique (modified Kirby-Bauer method) [19]. Zones of inhibition of azithromycin (15µg), erythromycin (15µg), clindamycin (2µg), linezolid (30µg), cefoxitin (30µg) were interpreted according to CLSI (2019) and ampicillin (10µg), vancomycin (30µg), novobiocin (30µg), teicoplanin (30µg) according to CLSI (2015) guidelines [20, 21]. Zones of inhibition of amikacin (30µg) and oxacillin (1µg) were interpreted according to EUCAST (2018) and Khan *et al*. respectively [22, 23]. Antibiotic discs were obtained from commercial source (Oxoid Ltd, Uk).

2.4. Control strain

Staph. aureus ATCC 25923 was used as control strain to assess the performance of the method.

2.5. Statistical Analysis

SPSS software (version 25) was used for data analysis. Chi-square test was used for analysis of categorical data. P value of < 0.05 was considered significant.

2.6. Molecular method [24, 25]

Polymerase chain reaction (PCR) was done for the detection of agr-I, agr-II, agr-III andagr-IV genes in biofilm forming *Staph. aureus.*

2.6.1. Bacterial pellet formation

A loop full of bacterial colonies from MHA media was inoculated into a microcentrifuge tube containing TSB. After incubation overnight at 37°C, the tubes were centrifuged at 4000 g for 10 minutes at 4°C and the supernatant were discarded and then the tubes containing bacterial pellets were kept at -20°C as pellets until DNA extraction.

2.6.2. DNA extraction

300 µl distilled water was mixed with bacterial pellet and was vortexed until mixed well. The mixture was kept in block heater (DAIHA Scientific, Seoul, Korea) at 100°C for 10 minutes for heating. After heating the tube was immediately kept on ice for further 5 minutes and then centrifuged at 14000 g at 4°C for 10 minutes. Finally, supernatant was taken into another microcentrifuge tube and was used as template DNA for PCR. Extracted DNA was preserved at -20°C for future use.

2.6.3. Mixing of master mix and primer with DNA template

Primers were diluted with Tris-EDTA (TE) buffer according to manufacturer's instruction**.** PCR was performed in a final reaction volume of 25 µl in a PCR tube, containing 12.5 µl of master mix (mixture of dNTP, Taq polymerase, $MgCl_2$ and PCR buffer), 1 µl forward primer and 1 µl reverse primer (Promega Corporation, USA) 2 µl extracted DNA and 10.5 µl of nuclease free water. After a brief vortex, the PCR tubes were centrifuged in a microcentrifuge for few seconds.

2.6.4. Amplification through thermal cycler

PCR assays were performed in a DNA thermal cycler (Eppendorf AG, Master cycler gradient, Hamburg, Germany). Each PCR run was comprised of preheat at 94°C for 10 minutes followed by 36 cycles of denaturation at 94°C for 1 minutes, annealing at 58°C for 45 seconds, extension at 72°C for 2 minutes with final extension at 72°C for 10 minutes.

2.6.5. Agarose gel electrophoresis

PCR products were detected by electrophoresis on 1.5% agarose gel was prepared with 1X TBE buffer (TrisBorate EDTA). For 1.5% agarose gel preparation, 0.18 gram of agarose powder (LE, Analytical grade, Promega, Madison, USA) was mixed with 12.5ml TBE buffer. Mixture was boiled for few minutes to dissolve and cooled to 60-70°C. A comb was placed in gel tray and poured the agarose gel. After solidification, comb was removed and 6µl of amplicon was mixed with 1µl loading dye on para film and then loaded into the well of agarose gel. 2 µl of DNA ladder was mixed with 1µl loading dye and was loaded into one well. Gel containing amplicon and DNA ladder were then placed on the electrophoresis tank having 1X TBE buffer for 35 minutes at 100 volts. Positive control and negative control was also loaded in separate well.

2.6.6. Staining and de-staining of the gel

After electrophoresis, the gel was stained with ethidium bromide (20 µl ethidium bromide in 200ml distilled water)

for 30 minutes. It was then de-stained with distilled water for 15 minutes.

2.6.7. Visualization and interpretation of results

The gel was observed under UV Trans-illuminator (Gel Doc, Major science, Taiwan) for DNA bands. The DNA bands were identified according to their molecular size by comparing with the molecular weight marker (100 bp DNA ladder) loaded in a separate lane. Samples showing the presence of corresponding bp band were considered positive for the presence of that organism.

2.6.8. Procedure of DNA sequencing

For sequencing of bacterial DNA, purification of amplified PCR product was done by using DNA purification kit (FAVOGEN, Biotech Corp). Purified PCR products of *Staph. aureus* were sent to 1st Base Laboratories, Malaysia for sequencing by capillary method (ABI PRISM 3500). BLAST analysis was performed to search for homologous sequences into the Gen Bank at www.ncbi.nlm.nih.gov.

2.6.9. Primers used in this study [26]

Gene	Primer sequence (5'-3')	Size (bp)
agr- I	Forward: 5-ATG CAC ATG GTG CAC ATG C-3	441
	Reverse: 5-GTC ACA AGT ACT ATA AGC TGC GAT-3	
agr- II	Forward: 5-ATG CAC ATG GTG CAC ATG C-3	575
	Reverse: 5-TAT TAC TAA TTG AAA AGT GGC CAT AGC-3	
agr- III	Forward: 5-ATG CAC ATG GTG CAC ATG C-3	323
	Reverse: 5-GTA ATGTAA TAG CTT GTA TAA TAA TAC CCA G-3	
agr- IV	Forward: 5-ATG CAC ATG GTG CAC ATG C-3	659
	Reverse: 5-CGA TAA TGC CGT AAT ACC CG-3	

3. Results

Out of 275 samples, 208 bacteria were isolated among which 45 (21.63%) *Staph. aureus* were identified.

Table I demonstrates detection of biofilm production by different methods. Among 45 *Staph. aureus*, TCP method were detected 32 (71.11%) and TM were detected 28 (62.22%) biofilm producers.

Table I: Detection of biofilm producers by TM and TCP method among isolated *Staph.aureus.* (N= 45)

	TM n (%)	TCP		P value
		BP n (%)	BN n (%)	
BP	28 (62.22)	26 (57.78)	2 (4.44)	>0.05
BN	17 (37.78)	6 (13.33)	11 (24.45)	
Total	**45 (100)**	**32 (71.11)**	**13 (28.89)**	

BP = Biofilm positive, BN = Biofilm negative, N = Total number of bacteria, n= Number of positive.

Table II demonstrates among 45 isolates of *Staph. aureus*11.11%, 28.89 % and 31.11% of them were strong, medium and weak biofilm producer by the TCP method and 6.67%, 22.22% and 33.33% of them were strong, medium and weak biofilm producer by the TM respectively.

Table II: Screening of the isolates of *Staph. aureus* for biofilm formation by TCP method and TM.(N= 45)

Biofilm formation	TCP n (%)	TM n (%)	P value
Strong	5 (11.11)	3 (6.67)	>0.05
Moderate	13 (28.89)	10 (22.22)	>0.05
Weak	14 (31.11)	15 (33.33)	>0.05
Total	**32 (71.11)**	**28 (62.22)**	

N = Total number of *Staph. aureus*, n= Number of biofilm producers

Table III shows comparison of antibiotic resistance pattern between biofilm-producing and non-biofilm-producing *Staph. aureus.* Among 32 isolated biofilm-producing *Staph. aureus* 53.13% were resistant to oxacillin, 46.88% were resistant to cefoxitin and ampicillin whereas out of 13 non biofilm-producing *Staph. aureus* 23.08% were resistant to oxacillin and ampicillin, 30.77% were resistant to cefoxitin. 21.88% biofilm-producers were resistant to vancomycin and 18.75% were resistant to linezolid but 61.53% non-biofilm-producers were resistant to vancomycin and linezolid. The resistance rate of biofilm producing *Staph. Aureus* were more in Azithromycin (62.50%), Clindamycin (59.38%), Erythromycin (56.25%), Oxacillin (53.13%), Cefoxitin and Ampicillin (46.88%) followed by Amikacin and Vancomycin (21.88%) and Linezolid (18.75%).

Table III: Comparison of antibiotic resistance pattern between biofilm-producing and non-biofilm-producing *Staph. aureus*.

Antimicrobials	**Biofilm producers (N=32) n (%)**	**Non-biofilm producers (N=13) n (%)**	**P value**
Azithromycin	20 (62.50)	11 (84.61)	>0.05
Clindamycin	19 (59.38)	11 (84.61)	>0.05
Erythromycin	18 (56.25)	13 (100.00)	>0.05
Oxacillin	17 (53.13)	3 (23.08)	>0.05
Cefoxitin	15 (46.88)	4 (30.77)	>0.05
Ampicillin	15 (46.88)	3 (23.08)	>0.05
Amikacin	7 (21.88)	5 (38.46)	>0.05
Vancomycin	7 (21.88)	8 (61.53)	<0.05
Linezolid	6 (18.75)	8 (61.53)	<0.05
Teicoplanin	3 (9.38)	8 (61.53)	<0.05

N = Total number of biofilm-producing *Staph. aureus*, n = Number of resistant bacteria

Table IV demonstrates the proportion of QS genes among biofilm producing *Staph. aureus*. Out of 32 biofilm-producing *Staph. aureus*, agr-I was the predominant one, followed by agr-III, agr-II and agr-IV.

Table IV: Proportion of agr genes among the isolated biofilm producing *Staph. aureus*. (N= 32)

QS genes	**Biofilm producing *Staph. aureus* n (%)**
agr-I	15 (46.87)
agr-II	2 (6.25)
agr-III	7 (21.88
agr-IV	2 (6.25)
No agr gene was found	6 (18.75)
Total	**32 (100.00)**

N = Total number of biofilm-producing *Staph. aureus*, n= Number of positive

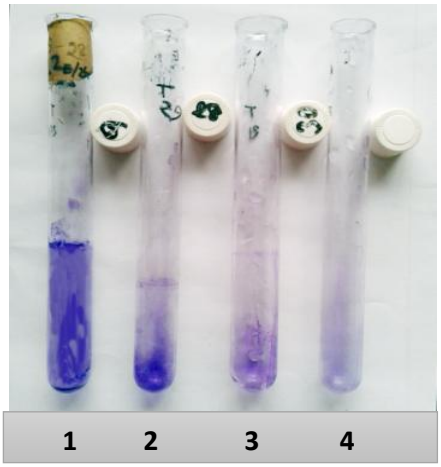

Figure 1: Photograph of detection of biofilm producing *Staphylococcus aureus* by tube method. 1, 2, 3 are strong, moderate and weak biofilm positive strain respectively and 4 is biofilm negative strain.

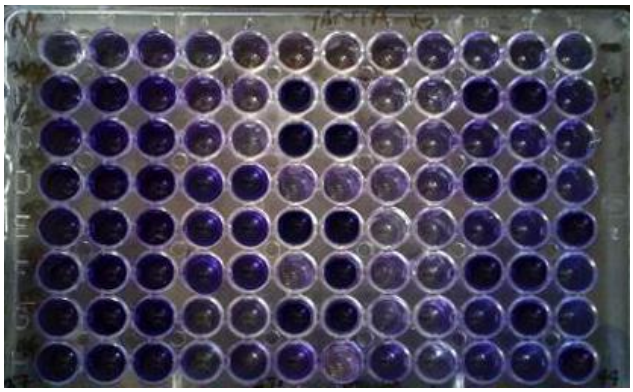

Figure 2: Photograph of screening of biofilm forming *Staph. aureus* by TCP method.

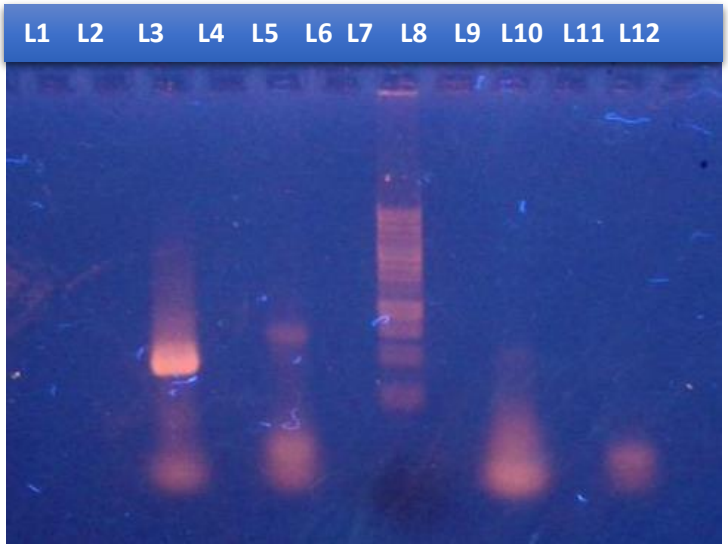

Figure 3: Photograph of gel electrophoresis: negative control without DNA (Lan 1), negative control *Staphylococcus aureus* ATCC 25923 (Lan 2), amplified DNA of 441 bp for agr-I gene (Lan 3), amplified DNA of 575 bp for agr-II gene (Lan 5), hundred bp DNA ladder (Lan 7), negative sample (Lan 12)

4. Discussion

Staph. aureus is the leading cause of biofilm-mediated life threatening infections, as biofilm production influences the efficacy of antibiotic therapy [2].

In the present study, 11.11% of the *Staph. aureus* were highly virulent showing strong adherence, 28.89% , 31.11% and 28.89% showing moderate, weak and non-adherence, respectively. This finding was almost similar to the related previous studies [27, 28]. Environmental factors like sugars (glucose or lactose) or proteases present in the growth medium, surface area, type of surface (rough/smooth), and porosity, charge of the surface and the genetic makeup of the *Staph. aureus* isolate affect biofilm formation [29].

Another study showed that 14.51%, 50.38% and 35.11% were strong, moderate and negative for biofilm formation respectively [30]. This difference in biofilm formation patterns among bacterial isolates may be due to difference in strain types, number of bacterial isolates, sample size, geographic locations and methodological variations to assess biofilm formation [31].

In this current study, antimicrobial resistance pattern was higher among non-biofilm-producing *Staph. aureus* than biofilm-producers. In this study, among biofilm-producing *Staph. aureus* 56.25%, 46.88%, 28.13% and 9.38% were resistant to erythromycin, cefoxitin, clindamycin and teicoplanin, respectively . This finding was nearly close to the previous studies [27, 32].

In our study, 46.88% biofilm-producing *Staph. aureus* were resistant to ampicillin. A previous study reported that 86.7% biofilm-producing *Staph. aureus* were resistant to ampicillin which was much higher than the present study [27]. The higher resistance of isolates to ampicillin may be attributed to the fact that it is the commonly used antibiotic for treatment of skin and nasal infections [33]. Differences in the antibiotic resistance patterns varies widely between different geographic regions. Many factors contribute to those patterns including local infection control programs implemented, antibiotic prescribing policies, epidemiology of the studied strains themselves and uncontrolled use of antibiotic in agriculture and livestock [34, 35].

In the present study, the total number of QS genes among 32 biofilm producing *Staph. aureus* isolates were 26(81.25%). Similar previous study reported that 50.82% QS genes were detected [36]. In this current study, among 32 biofilm producing *Staph. aureus* a majority (46.88%) of isolates belonged to agr-I, followed by agr-III (21.88%), agr-II (6.25%), and agr-IV (6.25%). This finding was nearly close to the previous studies [37, 38]. Another study reported that agr-II was the predominant one which was isolated from milk [39].This difference of obtained results may be due to differences of geographical location and source of isolation [36].

DNA sequence of amplified PCR product and translated nucleotide base sequence of agr-I showed point mutations and deletion including base substitution at multiple positions. Large and small deletions are the main source of gene-inactivating mutations followed by insertions/duplications and a considerable number of point mutation had been

detected, including truncation by nonsense and missense mutations [40, 41].

In terms of the annual burden of morbidity and mortality it imposes on society, *Staph. aureus* is potentially the most significant bacterial pathogen. This is particularly so in the case of chronic *Staph. aureus* biofilm infections. *Staph. aureus* is a clinically relevant pathogen due to its antimicrobial resistance and evasion of the host immune system [42]. So, biofilm detection may be included in routine laboratory tests for the better management of chronic or device associated infections.

Some studies have shown that some specific diseases were associated with specific agr types. One previous study showed that agr-I group was associated with invasive infections, especially bacteremia, agr-II group with invasive disease and agr-III group with noninvasive infection and TSST [43, 44, 45].

The resulting information would be very helpful for finding association of chronic *Staph. aureus* biofilm infections with agr specific types as well as in targeting agr for the development of anti-staphylococcal drugs.

5. Conclusion

The clinical isolates of *Staph. aureus* of hospitalized patients exhibit a high degree of biofilm formation. We can conclude from our study that TCP is a reliable method to detect biofilm forming microorganisms. TCP method can be recommended as a routine test for detection of biofilm producing bacteria in laboratories.

Higher rate of antimicrobial resistance is demonstrated by non-biofilm producers than biofilm-producers. Although biofilm-positive strains have a higher tendency to show resistance to oxacillin, cefoxitin, ampicillin compared to biofilm-negative strains but not statistically significant (>0.05).

Among biofilm-producing *Staph. aureus* agr-I gene was predominant than other agr genes.

6. Acknowledgement

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7. Conflict of interest

There is no conflict of interest.

8. Ethics approval

This study was approved by Research Review Committee (RRC) of Department of Microbiology and Ethical Review Committee (ERC) of Dhaka Medical College, Dhaka, Bangladesh (Reference number: MEU-DMC/ECC/2019/171).

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