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Intradermal Vaccination using Formaldehyde Inactivated Methicillin Resistant Staphylococcus Aureus induced Protective Immunoglobulin G Antibodies Targeting Protein of Specific Molecular Weight in Swiss Albino Mice

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Abstract

Background: Methicillin-resistant Staphylococcus aureus (MRSA) is one of the most common causes of hospital-acquired infections and is also a significant contributor to healthcare- and community-associated infections worldwide. The increasing resistance due to the presence of resistance genes, coupled with the irrational use of antibiotics, has worsened the clinical situation. This rising antimicrobial resistance highlights the urgent need for the development of an effective vaccine to prevent MRSA infections. Objective: The objective of this study was to identify a specific MRSA protein, on the basis of molecular weight, that induces defensive antibody conformation in serum and splenic cell culture supernatant following immunization with a formalin-inactivated whole-cell MRSA vaccine. Methodology: This was an animal study which was conducted in the Department of Microbiology at Dhaka Medical College, Dhaka, Bangladesh for a period of one year from January 2022 to December 2022. MRSA strains were obtained from various clinical samples and used to prepare a formalin-inactivated whole-cell vaccine. Fifteen Swiss albino mice were immunized intradermally with three boluses of this vaccine. Fourteen days after the third cure, the mice were challenged intradermally with live MRSA and covered for another 14 days. Sera were collected from tail blood, and splenic cell culture supernatants were gathered. The MRSA antigens used in the vaccine were sonicated and separated using SDS- PAGE electrophoresis according to molecular weight. ELISA was used to descry and quantify the antibodies in both serum and splenic cell supernatants that specifically bound to the MRSA antigens. Results: Following MRSA challenge, 100% of the immunized mice survived the 14-day observation period, indicating a strong protective response. ELISA results revealed that both pre- and post-challenge serum samples had significantly higher IgG optical density values compared to the control group. The highest antibody response was observed against the antigen eluted from the SDS-PAGE band corresponding to a molecular weight range of 95–130 kDa, in both serum and splenic cell culture supernatant. Conclusion: Formalin-inactivated intradermal immunization with MRSA generated a strong protective immune response in Swiss albino mice, targeting a specific antigen within the 95 to 130 kDa molecular weight range. This antigen may be a promising candidate for the development of a novel and effective MRSA vaccine.

Keywords: Antibody, Formalin inactivation, Immunization, MRSA, SDS-PAGE

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Introduction

Staphylococcus Aureus is a common inhabitant of the human body, belongs to the family *Staphylococcaceae*, often found on the skin and in the nose. It is a notorious pathogen capable of causing a wide range of infections, from common skin infections

to life-threatening systemic disease. With versatile virulence, it poses a significant challenge to current antibiotic treatments due to its ability to develop resistance mechanisms against a variety of clinically relevant antibiotics. Methicillin resistance in *Staphylococcus aureus* arises from the horizontal transfer of the *mec*A gene. Beyond producing a wide range of enzymes and toxins, the bacterium also forms biofilms, which contribute to its remarkable ability to withstand most currently available antibiotics. Treatment options are limited due to evolving resistance mechanisms, increasing morbidity, mortality, and costs[2].

Treatment of MRSA with vancomycin and increased empirical use of this antibiotic provided the antibiotic pressure associated with the emergence of vancomycin-resistant enterococci and vancomycin-intermediate-resistant *staphylococci*[3]. Vaccination represents one of the most powerful strategies against antibiotic resistance. Disease forestallment by vaccination lowers use of antibiotics. The most considerably tested vaccine against *Staphylococcus aureus*, which is a capsular polysaccharide-grounded vaccine known as StaphVAX, showed pledge in an original phase 3 trial, but was set up to be ineffective in a definitive trial, leading to its development being halted. Likewise, a mortal IgG medication known as INH-A21 (Veronate) with elevated situations of antibodies to the *staphylococcal* face adhesins *Clf*A and SdrG made it into phase 3 testing, where it failed to show a clinical benefit.

Several new antigens are being tested for implicit addition in a *staphylococcal* vaccine, including cell wall-anchored adhesin proteins and exotoxins[4]. For the construction of effective vaccines, it is critically significance to identify all antigens immunologically honored by a patient population infected with a pathogen[5]. A successful approach used to identify the stylish antigen campaigners for vaccines against pathogens is the use of immunoproteomics, which is grounded on a two-dimensional electrophoresis[6]. Extensively employed in biochemical examinations, Sodium Dodecyl Sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is a promising separation system for structural proteomics and an effective approach for protein separation in a straightforward procedure[7]. Using SDS-PAGE, certain native proteins can be uprooted in their pure form from cell lysates. This type of refined antigen constantly elicits favorable antibody responses[8]. ELISA test has always been considerably used for serological diagnosis and evaluation of immunity in vaccinated animals[9].

In sero-monitoring of vaccinated individuals and identification of antigen-antibody commerce in them, this system provides both perfection and specificity*10*.

In Bangladesh, there has been no previous attempt to explore MRSA antigens capable of stimulating defensive antibodies. Hence, the present work was conducted to identify protein antigens and to estimate the induction of defensive antibody responses through intradermal immunization with formaldehyde-inactivated MRSA using a murine infection model.

Methodology

Study Design and Setting: This research took place at Dhaka Medical College's Microbiology Department in Bangladesh from November 2022 to June 2023. To develop a vaccine, bacteria from various samples were grown in Blood agar medium for 24 hours at 37°C.

Animal Preparation: Fifteen 6 to 8-week-old female Swiss-albino mice were obtained from icddr,b's Animal Resources Facility and kept in precise pathogen-free environments in Dhaka Medical College's animal house.

Preparing MRSA Vaccine: Group-1 received a vaccine made from MRSA isolated from pus, wound swabs and blood.

Steps for Vaccine Preparation: *Staphylococcus aureus* (MRSA) was cultured in trypticase soy broth (TSB) at 37 °C for 24 hours under aerobic conditions. Following incubation, the bacterial culture was harvested by centrifugation, and the cell pellet was washed twice with phosphate-buffered saline (PBS) to remove residual media components. The bacterial suspension was then treated with 3% formalin, prepared from a 37% stock solution, and incubated at 37°C for 2 hours to ensure complete inactivation. After treatment, the cells were thoroughly washed and resuspended in PBS to obtain a final concentration of 1.5 × 10^8 CFU/ml for subsequent experimental use[11].

Immunization Schedule: Group-1 received three intradermal immunizations with 20 µl of the formaldehyde-inactivated solution (2X10^7 CFU/ml) on days 0, 14, and 28. Group-2, a control group, received 20 µl of sterile PBS, and group-3 remained uninoculated.

Blood Collection for ELISA: Blood was obtained from the tail tone of mice on days •13, 27, and 41post-primary immunization. The tail was gently extended and disinfected with 70 ethanol prior to sectioning. Using a sterile 22 FR scalpel, a 2-mm member was gutted from the distal tip of the tail, and

roughly 50 µl of blood was collected into a microcentrifuge tube containing 200 µl of phosphate-buffered saline (PBS) to achieve a 15 dilution. The adulterated blood was left upright at room temperature for 2 hours to allow clot conformation, followed by centrifugation at 3000 × g for 10 twinkles. The performing clear serum was precisely transferred to fresh microcentrifuge tubes and stored at - 20°C until farther analysis, in agreement with established protocols[12].

Intra-peritoneal Challenge: Fourteen days after the final inoculation, two groups of mice were given 3x108 live MRSA bacteria through a peritoneal depression challenge, after which they were covered for 14 days for signs of illness, including weight loss, languor, disinclination to eat, and death.

Separation of Mononuclear Cells from Spleen: Spleens were aseptically gathered from mice in Group-1(Experimental) and Group - 3 (Negative Control) to assess antibody product. Each spleen was placed in a petri dish containing 5 ml of complete RPMI medium (RPMI 1640 supplemented with 10 FBS, 200 U/ ml penicillin, and 200 µg/ ml streptomycin) under a sterile biosafety press. The spleens were gently disintegrated by pressing between two frosted glass slides, and the performing cell suspension was passed through a 70 µm nylon cell strainer. The filtrate was centrifuged at 350 × g for 10 twinkles at 4°C. The supernatant was discarded, and the cell bullet was resuspended in 5 ml of complete RPMI medium for posterior trials[13].

Determination of Viability of Splenic Mononuclear Cell: The splenic cell bullet was resuspended in 1 ml of PBS. An equal volume of 0.4 trypan blue result was mixed with the cell suspension to prepare stained sample. The admixture was incubated at room temperature for 3 twinkles. A drop of the stained cell suspension was also loaded onto a hemocytometer and examined under a microscope. Feasible (unstained) and non-viable (stained) cells were counted independently. The chance of feasible cells was calculated using the formula feasible cells = (total number of feasible cells per ml of aliquot/ total number of cells per ml of aliquot) × 100. The sample was considered applicable if ≥ 50 cells were set up feasible.

Antibody Discovery by ELISA: The presence of antibodies specific to MRSA in the mice's blood and splenic cell culture fluids was detected using an ELISA test to measure their optical density.

Sonication of Whole-Cell MRSA: About 100 microliters of distilled water adulterated bacterial bullets and were set away for 30 twinkles on ice. They were also sonicated at 20 kHz for 2 ten-alternate intervals. The adulterated result was centrifuged at 10,000 g for 20 twinkles to remove debris. Supernatants were transferred to a fresh microcentrifuge tube and stored at-20 degrees Celsius. To optimize the antigen, a checkerboard titration was performed. For ELISA, 10 micrograms of antigen were used[12].

SDS-PAGE Gel electrophoresis: A 10 resolving gel was prepared by successionally mixing distilled water, acrylamide/ bisacrylamide, Tris – HCl, SDS, 10 TEMED, and ammonium persulfate (APS), with TEMED and APS added incontinently before pouring to initiate polymerization. The gel was cast, leaving 2 cm for the mounding gel, which was prepared also with acclimated attention. Both gels polymerized fully within 30 twinkles The assembled gel was placed in the electrophoresis outfit, buffer chambers filled with running buffer, and samples along with molecular weight labels were loaded for electrophoretic separation. After electrophoresis, the gel was stained with Coomassie Brilliant Blue for at least 4 hours and destained overnight. Protein bands corresponding to 11 – 17, 26 – 34, 55 – 72, and 95 – 130 kDa were gutted and transferred to microcentrifuge tubes containing elution buffer. The gel slices were crushed, incubated overnight on a rotary shaker, and centrifuged at 10,000 × g for 10 twinkles. The supernatant containing eluted proteins was collected and stored for ELISA analysis[14-15].

Enzyme- Linked Immunosorbent Assay (ELISA): ELISA plates were carpeted with 100 µl/ well of antigen (10 µg/ ml) in bicarbonate coating buffer (pH 9.6) and incubated overnight at room temperature. Plates were washed twice with PBS and blocked with 200 µl/ well of skimmed milk in PBS, followed by incubation at 37°C for 1 hour 30 twinkles. Plates were also washed three times with PBS-Tween and formerly with PBS. Serum samples (100 µl/ well) and splenic cell culture supernatants (300 µl/ well) were added and incubated at 37°C for 1 hour, followed by late incubation at 4°C. The following day, plates were washed as described over, and 100 µl/ well of horseradish peroxidase (HRP)- conjugated anti-mouse IgG antibody (Thermo Fisher Scientific, USA), adulterated 15000 in PBS-Tween, was added and incubated at 37°C for 90 twinkles. After washing, 100 µl/ well of substrate result (50 µl tetramethylbenzidine 50 µl urea peroxide) was added. The response was stopped by adding 50 µl/ well of 1 M sulfuric acid.

Absorbance was measured at 450 nm using an ELISA plate reader (BioTek Inc., USA). The arrestment optical density (OD) value was calculated using the formula OD = M(mean) 2 × Standard divagation.

Data Analysis: All collected data were precisely collected, examined, and vindicated through repeated review. Errors and discrepancies were linked and corrected totally to ensure data delicacy and integrity. Statistical analyses, including t- tests and one- way ANOVA, were performed to compare datasets. Microsoft Excel 2020 was used for all analyses, and a p- value ≤ 0.05 was considered statistically significant.

Ethical Consideration: The study protocol and all animal experiments were approved by the Research Review, Ethical Review, and Animal Experimentation Ethics Committees of Dhaka Medical College.

Results

Survival rates of immunized mice challenged with MRSA demonstrated 100% survival, contrasting the 100% mortality observed in non-immunized mice. Immunized mice also exhibited a notable difference in serum Optical Density (OD) values upon inoculation, denoted by a p-value of less than 0.001 ($P < 0.001$) at each successive inoculation. In contrast, negative control sera exhibited a mean OD of 0.133, with a standard deviation of 0.003 and a cutoff value of 0.139. Immunized mice serum OD values demonstrated a range of 0.593-0.695 after the first inoculation, 1.295-1.385 following the second inoculation, and 1.529-1.933 after the third inoculation. Significant differences in OD values were observed in experimental and control mice sera

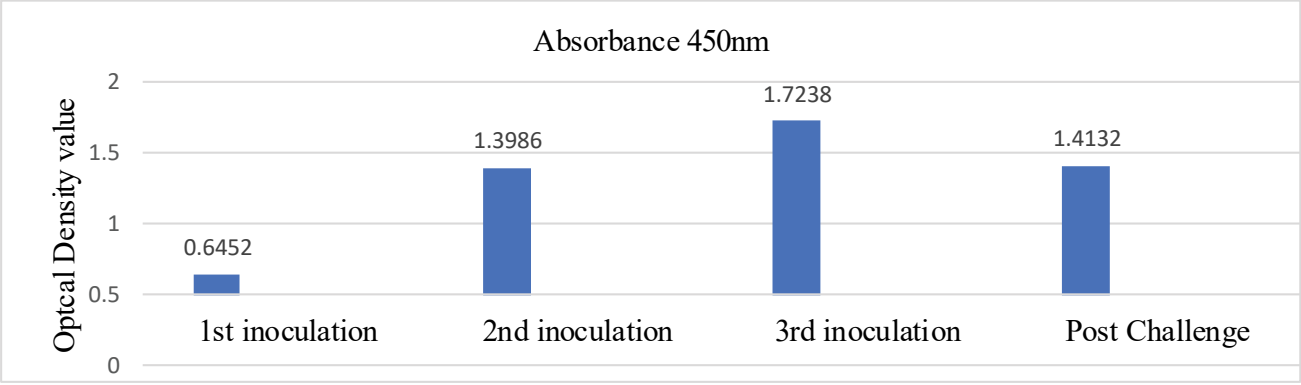

Figure I: OD values in serum samples from immunized group of mice after each inoculation and challenged by ELISA

Table 1: The OD value of IgG absorbance (450 nm) within the different inoculation schedule of experimental group interpreted by ANOVA (single factor)

Source of Variance	SS	Df	MS	F	P-Value
Between groups	3.105528	3	1.035176	82.73894	<0.0001
Within groups	0.200182	16	0.012511		
Total	3.35709	19			

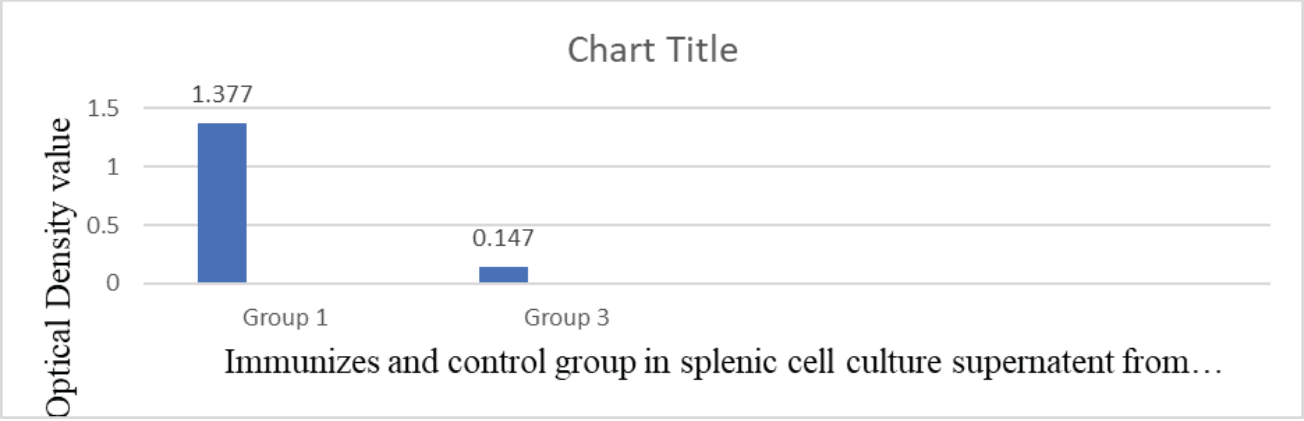

Figure II: Optical density (OD) of anti-Methicillin-Resistant Staphylococcus aureus (MRSA) antibodies in splenic cell culture supernatants.

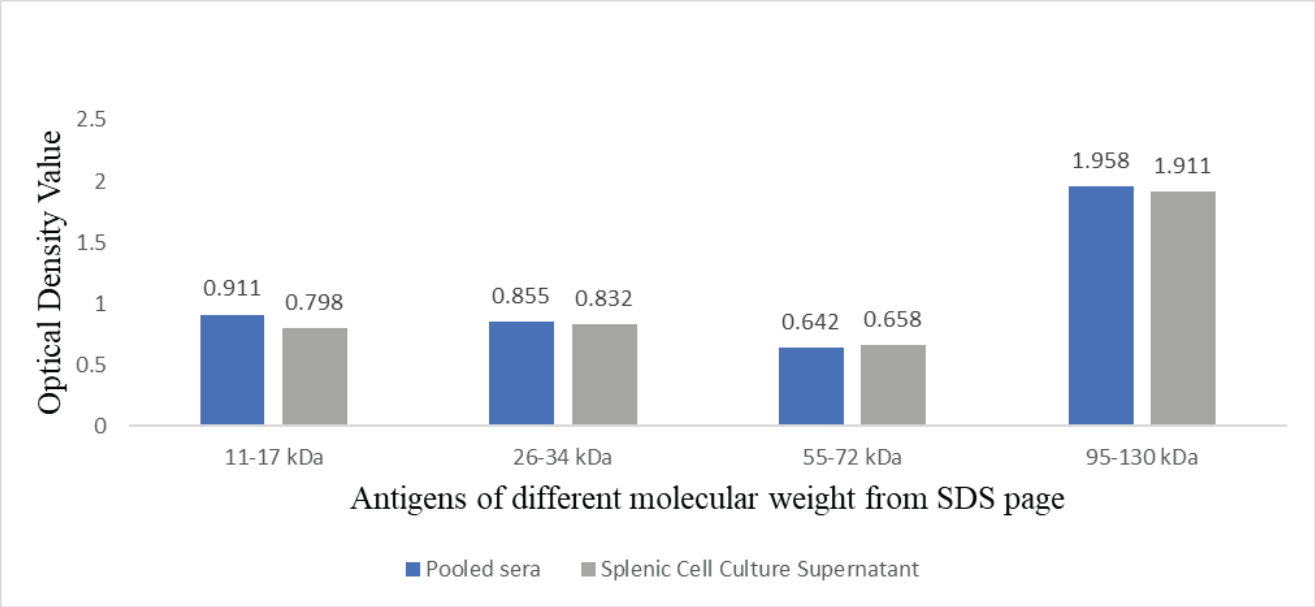

Figure III: OD values of the pooled sera and splenic cell culture supernatant of vaccinated group against eluded antigens of different molecular weight bands from SDS-PAGE

following each inoculation. The analysis of variance (ANOVA) revealed a notable immune response produced after each booster inoculation of inactivated whole-cell MRSA (Figure I).

There was also significant difference between OD values of cell culture supernatant of vaccinated mice group and negative control mice group ($P < 0.0001$) (Figure II).

Experimental group mice demonstrated significantly higher antibody production compared to the control group ($p < 0.0001$). ELISA measurements at 450 nm revealed that sera and splenic cell culture supernatants from vaccinated mice, exposed to inactivated MRSA antigens separated by SDS-PAGE, showed the highest absorbance against the 95–130 kDa protein band (OD = 1.958 and 1.911, respectively) (Figure III).

Discussion

Staphylococcus aureus is a significant contributor to both healthcare-associated infections (HAIs) and community-acquired infections (CAIs), particularly affecting hospitalized and immunocompromised individuals[16]. This pathogen possesses a broad array of virulence factors, including toxins, superantigens, and cell wall-associated exoproteins, which contribute to its pathogenic potential. The therapeutic management of infections caused by Methicillin-Resistant *Staphylococcus aureus* (MRSA) has become increasingly difficult due to the acquisition of multiple antibiotic resistance genes, including resistance to vancomycin the traditional drug of choice[17]. Globally, *S. aureus* bacteremia is associated with a case fatality rate ranging from 15% to 30% and accounts for approximately 300,000 deaths annually[18]. Thus, a comprehensive understanding of its epidemiology, virulence mechanisms, and clinical manifestations is essential for the development of targeted therapeutic and preventive strategies[19].

In light of escalating antimicrobial resistance, there is a critical and growing demand for alternative therapeutic approaches, particularly those based on immune modulation or vaccination, rather than antibiotics[20]. Intradermal (ID) vaccination offers promising advantages by eliciting a stronger immune response at lower antigen doses compared to conventional immunization routes[21]. In the present study, immunization with formaldehyde-inactivated MRSA conferred complete protection in the experimental mouse group, with 100% survival following bacterial challenge. In contrast, all non-immunized control mice succumbed to infection. Both pre- and post-challenge sera from immunized mice demonstrated significantly elevated antibody levels compared to controls, highlighting the efficacy of the vaccination. These findings align with previous preclinical studies demonstrating the protective effects of whole-cell inactivated vaccines in animal models such as mice and cattle[22].

Moreover, splenic lymphocyte culture supernatants from the vaccinated group also showed a significantly

higher concentration of antibodies relative to the unvaccinated control group, indicating robust systemic immune activation. ELISA, a standardized serological method, was employed to quantify antigen-specific antibody responses, revealing statistically significant elevations in vaccine-induced antibody titers. SDS-PAGE analysis of sonicated MRSA proteins identified four prominent molecular weight bands: 11 to 17 kDa, 26 to 34 kDa, 55 to 72 kDa, and 95 to 130 kDa. Subsequent ELISA testing using pooled sera from vaccinated mice demonstrated the highest absorbance at the 95 to 130 kDa protein band. Previous research has identified surface-associated bacterial proteins as key players in mediating S. aureus–host interactions, virulence, and immune recognition[23]. These proteins include autolysin, elongation factor Tu, protein A (SBI), alkyl hydroperoxide reductase, chaperone DnaK, superoxide dismutase, penicillin-binding protein 2, fructose-bisphosphate aldolase, and ABC transporter proteins.

Notably, immunodominant protein bands at 100, 70, 50, 30, and 23 kDa were consistently identified as highly immunoreactive across different S. aureus strains. In this study, the strongest antibody response was directed against the 95 to 130 kDa protein fraction, suggesting its immunodominance and potential utility as a vaccine candidate. The differences in protein band identification compared to other studies may be attributed to distinct selection criteria prioritizing additional factors beyond immunogenicity alone. Nevertheless, the consistent and heightened antibody response to the 95 to 130 kDa protein fraction emphasizes its significance in future vaccine development efforts targeting MRSA.

Conclusion

The growing threat of multidrug-resistant bacteria necessitates urgent action to enhance antibiotic stewardship and pursue alternative therapeutic options, including vaccines. Long-term immunity following vaccination is largely dependent on the activation of B-cell responses and subsequent antibody production. The current study demonstrates that formaldehyde-inactivated MRSA vaccination effectively induces protective immunity in mice, as evidenced by complete survival in the vaccinated group and universal mortality in unvaccinated controls. Although multiple antigens elicited antibody responses, the 95 to 130 kDa protein fraction emerged as the most immunogenic, with the highest IgG titers observed in both serum and splenic lymphocyte culture supernatants. These results underscore the potential of this high-molecular-weight antigen as a promising candidate for the development of a vaccine against MRSA. Future investigations should focus on the molecular identification and characterization of this antigen to further validate its role in protective immunity.

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None

Conflict of Interest
The authors have no conflicts of interest to disclose.

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Authors' contributions
Amena Khatun conceived and designed the study, analyzed the data, interpreted the results, and wrote up the draft manuscript; SM Shamsuzzaman contributed to the analysis of data and critical review of the manuscript; Mousumi Tania contributed to the analysis of the data, interpretation of the results, and critical review of the manuscript; Md. Ziaul Hassan was involved in the manuscript review and editing.
All authors read and approved the final manuscript.

Data Availability
Any inquiries regarding supporting data availability of this study should be directed to the corresponding author and are available from the corresponding author on reasonable request.

Ethics Approval and Consent to Participate
Ethical approval for the study was obtained from the Institutional Review Board. As this was a prospective study the written informed consent was obtained from all study participants. All methods were performed in accordance with the relevant guidelines and regulations.

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