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OPEN ACCESS ORIGINAL ARTICLE

Biofilm Producing Staphyloccusaureus: An Emerging Threat in Bloodstream Infections and Its Challenging Treatment Issues

Dr Amir Khan[1], Dr Rachna Tewari[1*]

[1] Department of Microbiology, Hamdard Institute of Medical Sciences and Research, Delhi, India

ABSTRACT

Background: Blood stream infection is a potentially life threatening infection with high mortality, which is predominantly caused by Staphylococcus aureus In the India. Methicillin classified MRSA is rising constantly in comparison of MSSA which makes Blood Stream infection more difficult to treat. On the other side BSI also classified on the basis of their transmission source, inside and outside of the hospital known as hospital acquired and community acquired BSI. **Objective**: The aim of our study was to determine, is the sensitivity testing if sufficient for treating BSI caused by S. aureus by understanding the difference between community associated and healthcare associated S. aureus infection in terms of methicillin resistance, biofilm producing ability and antimicrobial resistance pattern. As previous study says hospital acquired BSI is more resistant and having more ability to form biofilm on implants which makes them more difficult to handle. As there is a lack of next level treatment possibilities we need to find out all relative information to fill the gap between testing and treatment. **Methods**: This is a prospective study carried out on Staphylococcus aureus isolated from the Blood culture through outpatient and inpatient departments during period "between" January 2017 to December 2017. A total of 58 Staphylococcus aureus isolates were recovered from blood culture. All strains classified as MRSA and MSSA based on cefoxitin resistance. ViteK and Micro broth dilution method were used for antimicrobial resistance pattern and biofilm production respectively. **Results**: Out of 58 non-duplicate Staphylococcus aureus isolates, 21(36.20%) were community associated and 37(63.79%) were hospital associated, out of which 10(47.62%) and 22(59.46%) were Methicillin resistant and 11(52.38%) and 15(40.54%) isolates were Methicillin sensitive respectively. The biofilm producing ability was recorded as high biofilm producer, low biofilm producer and non-biofilm producer. On the basis of particular classification both community acquired and hospital acquired based MRSA (10, 22) was fund 4(40%), 13(59.09%) High biofilm producers, 1(10%), 4(18.18%) low biofilm producers and 5(50%), 5(22.73%) non biofilm producers. On a same hand Out of all 11 and 15 MSSA isolates of community acquired infection and hospital acquired infection were found as 2(18.18%), 4(26.67%) high biofilm producer, 2(18.18%), 4(26.67%) low biofilm producers and 5(50%), 7(46.67%) non biofilm producers. The antimicrobial resistance pattern difference been reported between hospital acquired and community acquired infection. The impact of biofilm producing ability been found on resistance pattern of all isolates. **Conclusion**: In our study most isolates were hospital associated. The presence of high resistance in IPD isolated MRSA isolates represent the impact of some other factors in hospital on antimicrobial resistance pattern, and after understanding the impact of biofilm production on resistance pattern helping somehow to fill the gap in between testing and treatment.

Key Words: *Biofilm, Methicillin-resistant Staphylococcus aureus, Methicillin-susceptible Staphylococcus aureus High-level, MRSA, MSSA, No biofilm, BSI, HBF, LBF, NBL, IPD, OPD*

***Corresponding Author**

Dr Rachna Tewari
Department of Microbiology, Hamdard Institute of Medical Sciences and Research, Delhi, India.

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INTRODUCTION

In India, BSIs due to Staphylococcus aureus is a leading cause of healthcare associated infection. Methicillin-resistant *Staphylococcus aureus* (MRSA) has been documented as an important cause of hospital infections all over the

world. However, MRSA has shown lot of changes in the its epidemiology as MRSA is now being isolated from community patients or people with no risk factors [1]. It is termed as community-associated MRSA (CA-MRSA) and have been reported from different community populations worldwide [2-4]. Having been implicated for potentially life threatening invasive infections, *Staphylococcus aureus* is one of the common agent of bloodstream infections. Blood stream infection (BSI)/bacteremiais defined as one or more positive blood cultures presenting with systemic signs of infection like fevers, chills, and/or hypotension. Both type of BSIs have been prevailing, community acquired or hospital acquired [5] and presented with detrimental clinical outcomes [6]. MRSA has lot of impact on costs and mortality rates as treatment is difficult [7-9].

CA-MRSA strains are clonal entities that differ from hospital-acquired MRSA (HA-MRSA) strains. These strains are those isolated outside hospital or within 48 hours of hospital admission, and if it was from a person who had not been hospitalized within two years before the date of MRSA isolation [10]. CA-MRSA and HA-MRSA are resistant to methicillin (and all beta-lactam antibiotics), but shows differences in epidemiology, lab characteristics, clinical aspects, and strategies for management. Spread of infection is not easily preventable as management of these multi drug resitant strains is challenging for the clinicians. Also 20–40% mortality at 30 days has been reported despite an appropriate treatment [11].

S. aureus infections have emerged as dangerous pathogens and costly to treat [12]. MRSA isolates are multidrug-resistant (MDR) as they show resistance to macrolides, tetracycline, aminoglycosides, chloramphenicol, and fluoroquinolones along with beta lactams, commonly used for their treatment [13].

Ability to form biofilm has also been found responsible for increased resistance against antimicrobial. It is assumed that biofilms contribute to >80% of infections in humans [14]. Colonization of medical surfaces, such as catheters and other devices, leads to healthcare-associated blood stream infections. Biofilm formation is a virulence factor in bacterimia because bacterial cells in biofilm are more resistant for antibacterials and host immune factors [15]. The aim of this study was to evaluate the capacity of biofilm producer and non producer MRSA to cause infections in community or hospital associated blood stream infections and impact on antimicrobial resistance pattern to improvise testing and treatment. Although Screening, testing and antibiotics have been upgraded, MRSA remains a major etiological agent for bacterimia. It is challenge to manage, especially in patients at high risk of complications. Therefore the main aim of this study was to investigate the capacity of clinical strains of *S. aureus* to form biofilms and impact on antimicrobial resistance pattern to fill the gap between testing and treatment.

MATERIAL AND METHODS

Study Type- Cross Sectional Study.
Inclusion Criteria- All S. aureus isolated blood samples.
Sample Size- Sample Size was calculated with the help of confidence level, confidence interval and targeted population by using survey software. Where confidence level was 95%, confidence interval was 5% and the targeted population was 60000. As study was conducted at a tertiary care hospital having patients from highly crowded area between 10 kms.
Ethical Clearance:- Ethical clearance been taken from IEC of Jamia Hamdard.

MRSA – How to isolate and confirm in lab?

All the samples obtained in the microbiology laboratory were processed as per standard operative procedures for routine isolation and identification of *Staphylococcus aureus*. Positive blood cultures were taken out from Bactalert and subcultured on 5% sheep blood agar and MacConkey agar media and incubated at 37°C aerobically. The growth was identified as *Staphylococcus aureus* by using conventional biochemical methods and Vitek - 2 automated identification system according to manufactures manual [16]. Strains were stocked in Di-methylesulfoxide (DMSO) for short time storage and at -80^{o}C for long term storage storage in deep refrigerators.

MRSA – Detection in lab.

MRSA was detected by using cefoxitin (30 µg) disc as per Clinical and Laboratory Standards Institute (CLSI) guidelines [18]. S. aureus strains were inoculated on nutrient agar and incubated aerobically for 6-8 hours. A suspension was made from growth of these strains. Turbidity was matched with 0.5 Mac Farland Standards. A lawn culture was prepared on the plate of Mueller-Hinton agar (MHA). Cefoxitin discs were placed and incubated for 24 hours aerobically at 35°C. After incubation the plates were examined for zone of inhibition around the cefoxitindisc. Zone of inhibition ≥22 mm was read as sensitive and not included in the study, whereas ≤21 was reported as resistant and confirmed as MRSA and included in the study.

Antimicrobial susceptibility testing of MRSA

Kirby Bauers disc diffusion method was used to measure antimicrobial susceptibility testing and MICs was confirmed byVitek 2 compact system. Antibiotics (Hi Media) tested were: benzylepenicillin, oxacillin, clindamycin, cotrimoxazole, erythromycin, ciprofloxacin, linezolid, vancomycin and tigecycline [17]. For Vitek 2 suspension was prepared and interpreted as per manufacturer instruction manual.

Detection of biofilm production - Microtiter plate (MTP) method [18]

Brain heart infusion broth supplemented with 2% glucose and 2% sucrose was inoculated with MRSA isolates and inubated for 6-8 hrs at 37°C. The growth was diluted 1:100 in medium, and sterile, 96 well micro titer plate was inoculated with 150 µl of this cell suspension and incubated at 37°C for 48 hours without shaking. Thereafter wells were washed three times, with 300µl of distilled water. Subsequently plates were dried and stained with 300µl of 2% crystal violet solution in water for 45 min. Washing was repeated in the same manner.

Wells were destained by adding 200µl of ethanol-acetic acid (95:5, vol/vol) so that quantitative analysis of production of biofilm can be done. From each well 100 microliters was transferred to a new microtiter plate, and Optical density; OD, of crystal violet present in the destaining solution was measured at 570 nm using a micro titer plate reader., uninoculated medium was ss a control. The mean OD_{570} value from the control wells was subtracted from the mean OD_{570} value of tested well. Picture -1

Picture 1: Microtitre plate showing biofilm production

Table 1: Optical Density and biofilm production interpretation

O.D.	Biofilm
<0.20	Negative
0.20-0.50	Positive (Low Biofilm Formation)
>0.50	Positive (High Biofilm Formation)

Statistical analysis

Microsoft excel sheet was used to calculate and record the data. Data were read, calculated and analyzed statistically by SPSS software. For calculation of significant levels Chi-square test was used. Statistically significant *P* values were taken as <0.05.

RESULTS

Distribution of Staphylococcus on the basis of area of isolation (community versus hospital)

A total of 58 *Staphylococcus aureus* were obtained from blood culture. 37(63.79%) strains were isolated from the hospital and 21(36.21%) were community acquired strains. Table 2

	Sample Colected (n=58)	
Healthcare Acquired	37	63.79%
Community Acquired	21	36.21%

The prevalence of MRSA isolation from blood samples was quite high in HA strains (59.46%) than CA -strains (47.62%). On the contrary prevalence of MSSA was high in CA strains (52.38%). Table 3

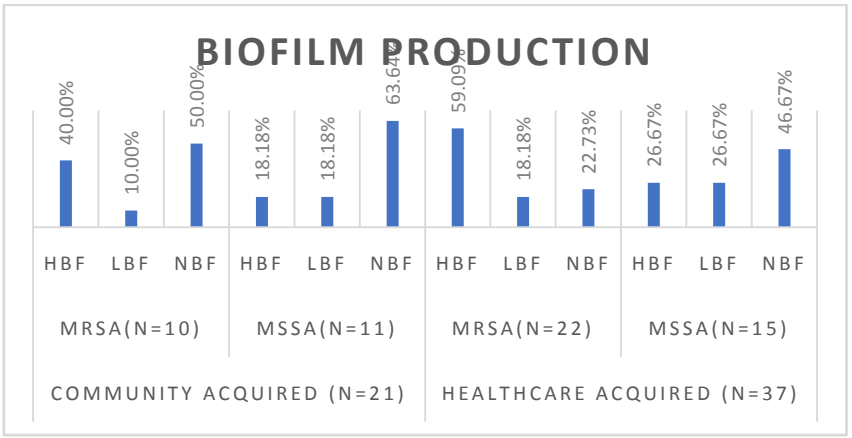
Table 3: Prevalence of MRSA and MSSA from blood samples in community and hospitals

	HA- SA (n=37)	
MRSA	22	59.46%
MSSA	15	40.54%
	CA - SA (n=21)	
MRSA	10	47.62%
MSSA	11	52.38%

Table 4: Biofilm Production in different strains

Biofilm Production				
CA-MRSA (n=21)	**MRSA(n=10)**	**HBF**	4	40.00%
		LBF	1	10.00%
		NBF	5	50.00%
	MSSA(n=11)	**HBF**	2	18.18%
		LBF	2	18.18%
		NBF	7	63.64%
HA-MRSA (n=37)	**MRSA(n=22)**	**HBF**	13	59.09%
		LBF	4	18.18%
		NBF	5	22.73%
	MSSA(n=15)	**HBF**	4	26.67%
		LBF	4	26.67%
		NBF	7	46.67%

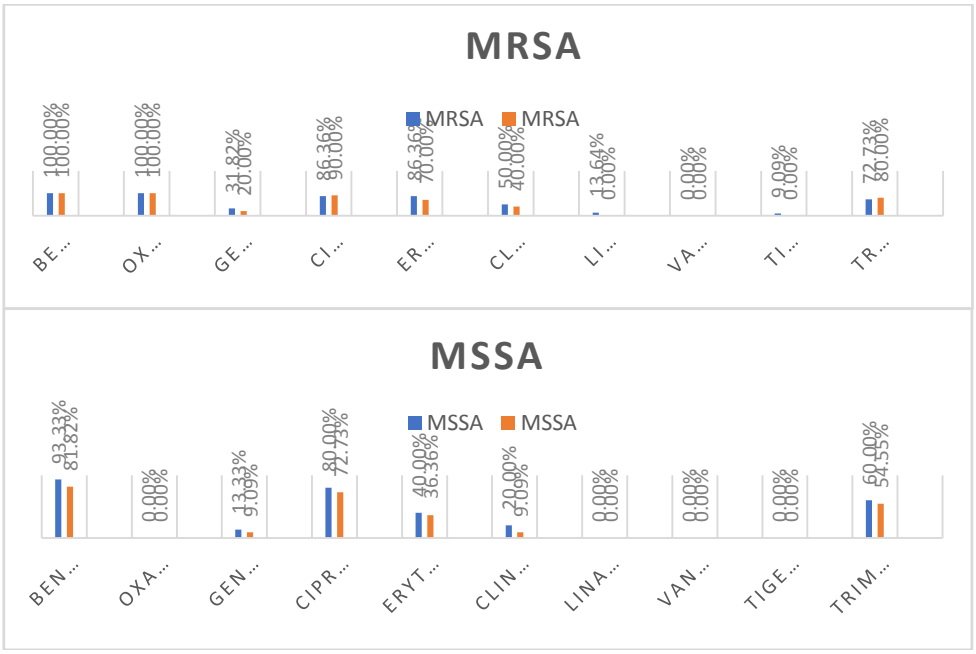
Most of the high biofilm producers were found in HA- MRSA (59.09%) followed by CA-MRSA (40%), HA- MSSA (26.67%) and then CA- MSSA (18.18%). Low biofilm producers are was found to be higher in HA- MSSA (26.67%) and CA – MSSA (18.18%), Non biofilm producers were more in CA- MRSA than HA - MRSA. Resistance was higher in biofilm producers.



Graph 1: depicts the comparison of biofilm production in CA and HA, MRSA and MSSA strains

Antimicrobial susceptibility of MRSA and MSSA isolates- Graph 2

In comparison of antimicrobial pattern of MRSA and MSSA isolates, we found mrsa were more resistant for in Gentamycin, Erythromycin, Clindamycin, Linezolid and Tigecycline. The HA- MRSA strain were found highly resistant than compared to CA-MRSA Similarly HA-MSSA were found to be more resistant than CA-MSSA. We found high MRSA strains were more resistant for Gentamycin, Erythromycin, Clindamycin, Linezolid and Tigecycline. The ratio of resistance for HA- MRSA to CA – MRSA for gentamycin was 31.82%: 20% and for HA-MSSA to CA-MSSA was 13.33%: 9.09%. Similarly for Erythromycin 86.36%: 70% in MRSA and 40%, 36.36% in MSSA. For clindamycin 50%: 40% for MRSA and for MSSA - 20%, 9.09%. Resistance for Linezolid (13.64%) and Tigecycline (9.09%) was found in HA MRSA only. But none of the MSSA strains were resistant for Linezolid and Tigecycline .All the strains were sensitive for vancomycin.



Graph 2: Comparision of antimicrobial susceptibility of MRSA and MSSA isolates-

DISCUSSION AND CONCLUSION

The inclusion criteria matched 58 cases of BSI between in a period of one year used in this study. As seen in the results section, an overwhelming percentage (63.79%) of all S aureus BSI cases were hospital associated and (36.21%) were community associated. As all the blood samples from IPD were taken after 3 days of hospital stay. Which reflects the higher hospital associated infection. The study found, the number of MRSA cases from inpatient department (59.46%) were more than MRSA been found in outpatient department (47.62%). On a same side antimicrobial resistance pattern, biofilm producing ability also been found higher in IPD isolated MRSA isolates and the comparison between biofilm producing IPD isolated MRSA then other isolated the same strains been found higher resistance rate for Erythromycin, Clindamycin, Tigecyclin and linezolid.

We have found similar kind of results with few studies i.e Athena P. Kourtis at al [18]. and whereas mostly studies shows the higher BIS caused by Staphylococcus aureus related to community associated instead of hospital, i.e. Yasmin, M., El Hage at al. [19] As in our study biofilm forming MRSA infection was mostly associated with hospital infection which is similar to the study conducted by Jeong-Ok Cha at. Al [20] which represent the hospital serves more suitable environment for biofilm production may be related to various healthcare associated risk factor. The Hospital associated MSSA also produces higher biofilm in comparison to other leading the result in a right direction. However, among the community associated cases, approximately 45% of the cases revealed the biofilm producing ability suggests the biofilm formation may be troublesome in community as well as healthcare.

S. aureus has been reported as most common cause of BSI infections. Its way up to high mortality due to increasing antimicrobial resistance is being documented every day in India. Genetic modifications are held responsible for increasing antimicrobial resistance, and other virulence factors. In our study also, Staphylococcus aureus was found most prevalent etiological agent of blood stream infections. It was found to be causing more infections in hospital (63.79%) than in community (36.21%). MRSA strains were more prevalent in hospital. Whereas MSSA were isolated more from community samples. Hospital strains were more resistant for antibiotics than community strains no matter it was MRSA or MSSA. An inverse association was found between biofilm production and antimicrobial sensitivity. Biofilm production was also noted to be higher in HA- strains than CA –strains and so the resistance for antibiotics, making treatment difficult and costly.

In contrast to our study many studies documented that the higher BIS caused by Staphylococcus aureus was community associated instead of hospital [19]. although few studies came out with similar results as of ours [20]. Short duration and small sample collection was the limitation of this study. The ability of biofilm production is found to be closely related to bacterial survival and virulence and so chronic type of infections are now believed to have some link with biofilms formation [14]. Biofilms are good to protect S. aureus from host defenses and antibiotics and are consequently help in its pathogenesis .Similar to our studies biofilm-forming MRSA infection has been reported to be highly associated with nosocomial infection by other studies [9, 11]. It might be due to hospital environments and healthcare-associated risk factors like catheters which are considered to be more suitable for biofilm formation in BSIs. Significant risk factors like the presence of invasive devices and prior hospitalization, antibiotic use and MRSA colonization were more common in biofilm-forming isolates. However, among the community cases, nearly half of the

isolates were interpreted as biofilm-producers. This conveys that biofilm formation is a challenge not only for HA-MRSA but also for CA-MRSA as well.

Biofilm matrix act as physical barrier between bacteria and antimicrobial and bacteria change phenotypically in this matrix. As a result, biofilm-associated infections are notoriously difficult to eradicate. Most chronic MRSA infections are due to this biofilm state which has important role in their pathogenesis [15]. To overcome the morbidity and mortality of S. aureus associated BSI it's important to fill the gaps between testing and treatment. It is equally important to focus on antibiotic stewardship to reduce the emerging pressure for emerging more resistant pathogenic strains of Staphyloccus aureus in BSIs.

The best method for treating a biofilm-related infection is by preventing initial infection by proper infection control methods. Biofilms significantly increase antibiotic minimum inhibitory concentrations (MICs) compared to cells in the free state [20]. Biofilm-associated infections are most commonly treated by vancomycin. It has been reported that the MIC is 10-times higher for biofilm-bound cells than for planktonic, free-floating cells [21]. Then Daptomycin can be tried, considered effective at treating even VRSA biofilm-related infections and considered most effective antibiotics at clearing S. aureus from an existing biofilm [22].

It is essential to develop valuable tools like set up quality assurance programmes to control the infections related to catheters and devices, as well as training and awareness programmes for patients and staff, S. aureus vaccine for haemodialysis patients that contribute to reducing blood stream infection infection [23, 24].

As treatments are only effective for planktonic cells or acute infections, need to develop new therapeutic strategies capable of clearing S. aureus in the biofilm state should be fulfilled. Efforts for development of biofilm inhibitors are still in its infancy. While many new approaches such as tiny molecules to prevent formation of biofilm, enzymes to weaken biofilm matrix, and antibodies and vaccines that target specific biofilm stages have been in progress but lack clinical validation [15]. Host defense mechanisms can also be targeted for treatment. Studies have demonstrated that human milk oligosaccharides (HMOs), modulate growth and biofilm production for several bacterial pathogens, including MRSA [25].

To conclude, antimicrobial sensitivity alone is not sufficient for treatment of BSIs due to biofilm production in S. aureus and cant solve this threat but prevention by proper screening, sequencing, hospital infection programmes, testing for biofilm production and continous research for new strategies to control biofilm production is an urgent need of hour to control BSIs in hospital and community.

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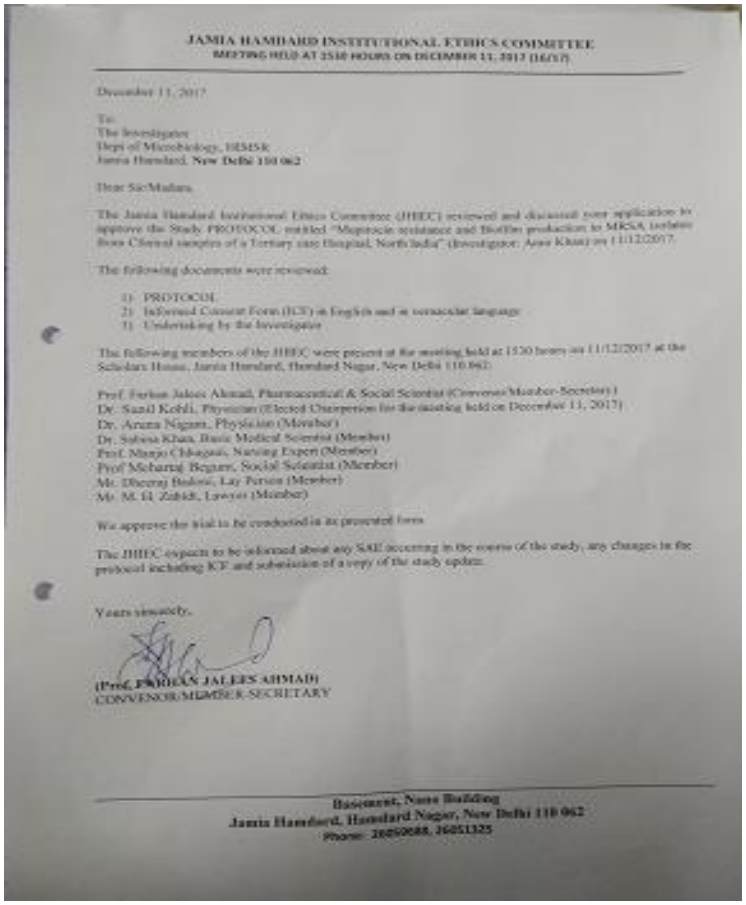

JAMIA HAMDARD INSTITUTIONAL ETHICS COMMITTEE
MEETING HELD AT 1530 HOURS ON DECEMBER 11, 2017 (11/12/17)

December 11, 2017

To
The Investigator
Deptt of Microbiology, HSMSR
Jamia Hamdard, **New Delhi 110 062**

Dear Sir/Madam,

The Jamia Hamdard Institutional Ethics Committee (JHIEC) reviewed and discussed your application to approve the Study PROTOCOL entitled "Magnesin resistance and Biofilm production in MRSA isolates from Clinical samples of a Tertiary care Hospital, North India" (Investigator: Amir Khan) on 11/12/2017.

The following documents were reviewed:

1) PROTOCOL
2) Informed Consent Form (ICF) in English and in vernacular language
3) Undertaking by the Investigator

The following members of the JHIEC were present at the meeting held at 1530 hours on 11/12/2017 at the Scholars House, Jamia Hamdard, Hamdard Nagar, New Delhi 110 062:

Prof. Farhan Jalees Ahmad, Pharmaceutical & Social Scientist (Convenor/Member-Secretary)
Dr. Sunil Kohli, Physician (Elected Chairperson for the meeting held on December 11, 2017)
Dr. Aruna Nigam, Physician (Member)
Dr. Sabina Khan, Basic Medical Scientist (Member)
Prof. Manju Chhagani, Nursing Expert (Member)
Prof Meharaj Begum, Social Scientist (Member)
Mr. Dheeraj Bakshi, Lay Person (Member)
Mr. M. H. Zahidi, Lawyer (Member)

We approve the trial to be conducted in its presented form.

The JHIEC expects to be informed about any SAE occurring in the course of the study, any changes in the protocol including ICF and submission of a copy of the study update.

Yours sincerely,

(Prof. FARHAN JALEES AHMAD)
CONVENOR/MEMBER SECRETARY

Basement, Nasir Building
Jamia Hamdard, Hamdard Nagar, New Delhi 110 062
Phone: 26059688, 26051325