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RESEARCH ARTICLE OPEN ACCESS

Urine Microscopy Score and Neutrophil Lymphocyte Ratio at Presentation are Good Biomarkers of Acute Kidney Injury in Patients with Upper Urinary Tract Infection when Assessed in Correlation with Virulence Factors of *Escherichia coli* and Blood Group Secretor Status

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Abstract

Acute kidney injury (AKI) is a leading cause of morbidity in urinary tract infection (UTI). We aimed to identify simple biomarkers and bacterial virulence factors associated with AKI in a setting of upper UTI due to uropathogenic *Escherichia coli* (UPEC). We designed a cross-sectional study to analyse biomarkers of AKI in upper UTI patients. A total of 2758 patients presenting to a tertiary care center with symptoms of upper UTI were assessed for the presence of diabetes mellitus, AKI, blood group non-secretors, urine microscopy and hemogram. 300 patients with UPEC in bacterial culture were studied for bacterial virulence factors by phenotypic and genotypic methods and the patients were followed up for a minimum period of two weeks. Patients with diabetes mellitus, non-secretors, Urine microscopy (UM) score > 2 and higher (> 3.9) neutrophil-lymphocyte ratio (NLR) at initial presentation, were found to be significantly associated with AKI at presentation and predicted AKI at 7th and 14th day follow up. They were also significantly associated with need and increased duration of hospitalization. There was no association of virulence factors of UPEC with diabetes mellitus, AKI or need for hospitalization. However, virulence factors had a significant association (p<0.001)with non-secretors. UM score and NLR are simple tools to identify AKI at presentation and predict AKI during follow-up and the need for hospitalization. Patients with diabetes mellitus and non-secretors are also at higher risk of AKI. Non-secretors are significantly associated with both phenotypic and genotypic expression of virulence factors of UPEC.

Keywords: Acute Kidney Injury, Neutrophil lymphocyte ratio, Urine microscopy score, non-secretors, Virulence factors of Uropathogenic *Escherichia coli*

INTRODUCTION

Kidney disease international guidelines organization (KDIGO 2012) defined AKI based on "the elevation of serum creatinine and reduction in urine output".[1] Serial measurements of both have several limitations in the diagnosis of AKI and can lead to a significant delay in diagnosis of renal parenchymal injury. Upper UTI remains a major intrinsic cause of AKI. Unlike lower UTI, which presents early, lack of specific symptoms precludes the early diagnosis of upper UTI unless there is a high index of clinical suspicion.[2]

Early diagnosis of AKI remains the cornerstone to prevent the severity of the disease. Urine microscopy is a valuable non-invasive and highly sensitive biomarker for early prediction of AKI, but it is also one of the most under-utilized ones.[3]

Urine microscopy is a rapid bedside screening test and as well can be used to assess the severity of renal parenchymal involvement. In order to standardise the interpretation, Perazella had proposed a structured scoring system and found it to be useful in several of his studies in the assessment of AKI due to various causes.[4,5]

Another important minimally invasive and low-cost screening tool with significant utility in the diagnosis of AKI is the neutrophil-lymphocyte ratio (NLR). However, hemograms are repeated several times in a patient presenting with fever and symptoms of acute infection. Hence, a clear definition for the assessment of NLR value at a particular time point is critical to avoid confounders in its utility as a biomarker.[6]

AKI due to upper UTI, is significantly affected by the virulence of bacteria as well as the anti-bacterial resistance.[7] There are several virulence factors in UPEC which are attributed to the pathogenesis and impact the disease severity such as adhesion factors, toxin production etc.

UPEC strains exhibit both structural (as fimbriae, pili, curli, flagella) and secreted (toxins, iron-acquisition systems) virulence factors that facilitate invasion of the urothelium of patients and contribute to the disease.

Non-secretors are at a higher risk for bacterial adhesion and their association with virulence factors of UPEC is not well studied. Also, the majority of the studies utilize FUT2 gene polymorphisms for the study of secretor status and not the phenotypic expression. Gene polymorphisms vary in different demographics, leading to challenges in assessing the relation of

secretor status with bacterial virulence factors or disease manifestations.[8]

In addition, inflammatory response to UTI may worsen pre-existing renal insufficiency.[9] The study was designed to assess AKI in patients with upper UTI and association with bacterial virulence factors, secretor status and biomarkers such as NLR and UM score.

METHODOLOGY

The study setting was cross-sectional analytical in adults (>13 years), who presented with upper UTI in the Departments of Medicine and Urology in a referral hospital. It was a prospective study with evaluation of the patient at three-time intervals, at initial presentation, 7th and 14th day of follow up. The patients were enrolled in the study from June 2017 to June 2019, after informed ethical consent. All patients in the study were followed up for a minimum period of 14 days with the assessment of serum creatinine, urine output, blood glucose, complete hemogram and duration of hospital stay.

Upper UTI was defined based on "clinical symptoms viz., loin to groin pain and fever with rigors, in combination with or without lower urinary tract symptoms". Lower UTI was defined based on "increased frequency, urgency, dysuria, and suprapubic pain".[1] Those patients who had previously known renal diseases and contracted kidney on ultrasound examination suggesting end-stage renal disease (ESRD), were excluded to minimize confounders.

In our study population, very sick patients with bacteremia and features of sepsis (estimated by qSOFA score), hypertensives, known renal diseases and chronic kidney disease were excluded and we did not have patients with ICU admissions, multi-organ failure, renal replacement therapy or dialysis.

Midstream urine specimen was collected and the specimen was processed for microscopy and urine culture.[6]

Urine Microscopy Semi-quantitative scoring system (UM)

Sediment from centrifuged urine was transferred to the glass slide, cover-slipped and assessed at 40x magnification for the research of blood cells, renal tubular epithelial cells (RTEC), granular and hyaline casts. The number of RTEC and granular casts were scored as per Perazella scoring system[4] and a score of > 2 was taken as a cut-off for predicting AKI.

Phenotypic characterisation of Virulence factors of UPEC

UPEC has various virulence factors playing a significant role in the pathogenesis of UTI. Hemolysin production was identified by clearing the medium by hemolytic colonies in 5% sheep blood agar.[10] Type 1 and P fimbrial antigen was assessed by Mannose-sensitive and Mannose-resistant hemagglutination respectively by slide method.[10] Serum resistance was assessed by reduction in the viable count of bacteria which was incubated over a period of time by serum bactericidal activity.[11] Biofilm production was identified by Crystal violet colorimetric method using ELISA reader.[10]

Diabetic assessment

Chemiluminescence assay was used to analyse creatinine and sugar levels in serum from fasting as well as 2 hours post-prandial state for identifying patients with diabetes mellitus and AKI. Complete blood count and peripheral smear examination were performed using fully automated analyser (Beckman Coulter) followed by Leishman stain, and NLR was calculated from absolute counts.

Secretor status assessment by the adsorption-inhibition method

Adsorption-inhibition method was utilized to assess the secretor status of patients in saliva specimens, as enlisted in our previous publication.[12] Secretor status assessment was repeated twice and concordant results were taken for assessment.

DNA Extraction

Overnight growth of bacterial culture was processed in Luria Bertani broth. Manufacturer protocol was used for DNA isolation using a Qiagen isolation kit (QIAmp UCP Pathogen Mini kit No. 50214). Quantification of isolated DNA was performed using a Qubit 3.0 fluorometer with internal controls.

Real-Time Thermal Cycler

Genes coding for virulence factors of *E. coli* viz., type 1 fimbriae (*fimH*), P fimbriae (*papA*), serum resistance (*iss*), hemolysin (*hlyA*), cytotoxic necrotising factor (*cnf1*), curli fimbriae (*csgA*) and biofilm (*iutA*), were identified using

TaqMan probes designed by Helini Biomolecules, India, based on sequences from NCBI (Table 1), following the methodology as detailed in our earlier publication.[7] Real-time thermal cycler from ABI QS3 was utilized with in-built blanks (negative controls) and positive commercial controls for every run. Concordance with a mean ct value of three thermal cycler runs was used to identify virulence genes.

Statistical Tests

Pearson's Chi-square test was used to test the hypothesis on the association between categorical variables, diabetes mellitus and secretor status with AKI at various time intervals, the requirement for various durations of hospital stay and genotypic and phenotypic virulence factors of UPEC. Pearson's Chi-square test was also used to test the hypothesis on the association of biomarker UM score as a categorical variable with AKI at various time intervals. The association of NLR as a continuous variable with AKI at presentation was studied using the two-sample

Table 1. Primer and Probe sequence of genes for Real Time PCR

No.	Gene	Primer	
1.	*fimH*	Forward	TTTTGCGACAGACCAACAAC
		Reverse	GGTGACATCACGAGCAGAAA
		probe	AATAATGATGTGGTGGTGCCCACTG
2.	*papA*	Forward	CAGATATCTCTGGTGTGTTCAGT
		Reverse	GGAATAGTTGGAGCAGCATTTAC
		probe	TTATTGCCGGTGCGGTAGCTATGG
3.	*Iss*	Forward	CCGACAGCAGTAACACCAAA
		Reverse	ACCGAGCAATCCATTTACGA
		probe	CACTCATCATTTCTTCGTTTCGGGAA
4.	*hlyA*	Forward	AGAAACTCATTGGCCTCACC
		Reverse	GTACACTGCCTGCCTTTCCT
		probe	AAAGCGGGTAATAAATTAGGCGGCA
5.	*cnf1*	Forward	TTCTCTGGACTCGAGGTGGT
		Reverse	TGCGGTAATTTTGGGTTTGT
		probe	CCTATTCTGCAGTGACCGGATCTCC
6.	*csgA*	Forward	GCAGCAATCGTATTCTCTGGTAG
		Reverse	TATTACCGCCACCACCGT
		probe	TGGCAGGTGTTGTTCCTCAGTACG
7.	*iutA*	Forward	ATTCAGGGCGGCAAAGAG
		Reverse	GGCTGTCGGTACGTGAAGAG
		probe	ACTACGGTATGAATGTGCGTGGCC

Table 2. Association of diabetic and secretor status of the patient with AKI at presentation and at 14 days follow up

Diabetes mellitus	AKI at presentation		Pearson Chi^2 P-value	AKI at 14 days follow up		Pearson Chi^2 P-value
	Absent	Present		Absent	Present	
Absent	140(73%)	49(26%)		162(85.7%)	27(14.3%)	
Present	61(55%)	50(45%)	<0.001	78(70.3%)	33(29.7%)	<0.001
Total	201(67%)	99(33%)		240(80%)	60(20%)	
Secretor status	Absent	Present		Absent	Present	
Nonsecretor	104(56.8%)	79(43.17)		137(74.9)	46(25.1)	
Secretor	97(82.9%)	20(17.09)	<0.001	103(88.03)	14(11.97)	0.005
Total	201(67%)	99(33%)		240(80%)	60(20%)	

Table 3. Association of diabetic and secretor status of the patient with need and duration of hospital stay

Diabetic Status	Outpatient care (0 days)	Hospital stay (1-5 days)	Hospital stay (>5 days)	Pearson Chi^2 P-value
Non-Diabetic	37(19.6%)	112(59.3%)	40(21.1%)	
Diabetic	17(15.3%)	37(33.3%)	57(51.4%)	<0.001
Total	54(18%)	149(49.7%)	97(32.3%)	
Secretor status				
Nonsecretor	29(15.8%)	81(44.3%)	73(39.9%)	
Secretor	25(21.4%)	68(58.1%)	24(20.5%)	0.002
Total	54(18%)	149(49.7%)	97(32.3%)	

Table 4. Expression of Virulence factors and presence of genes in *E.coli* isolates of patients

Virulence Factors	Absent	Present	Virulence genes	Absent	Present
Hemolysin	222(74%)	78(26%)	*hlyA*	216(72%)	84(28%)
MSHA	237(79%)	63(21%)	*fimH*	238(79.3%)	63(20.7%)
MRHA	167(55.7%)	133(44.3%)	*papA*	174(58%)	126(42%)
Serum resistance	58(19.3%)	242(80.7%)	*Iss*	234(78%)	66(22%)
Biofilm production	104(34.7%)	196(65.3%)	*iutA*	170(56.7%)	130(43.3%)
			csgA	81(27%)	219(73%)
			cnf1	181(60.3%)	119(39.7%)

Note: MSHA- Mannose sensitive hemagglutination; MRHA- Mannose resistant hemagglutination.

Table 5. Correlation of Secretor status with phenotyping of virulence factors *E.coli* in UTI

Secretor Status	Hemolysin		Pearson Chi^2 P-value
	Absent N (%)	Present N (%)	
Non-Secretor	116 (63.4%)	67 (36.6%)	Pearson Chi^2 = 27.46
Secretor	106 (90.6%)	11 (9.4%)	P <0.001
Total	222	78	
MSHA			
Non- Secretor	135 (73.8%)	48 (26.2%)	Pearson Chi^2 = 7.73
Secretor	102 (87.2%)	15 (12.8%)	P = 0.005
Total	237	63	
MRHA			
Non- Secretor	74 (40.4%)	109 (59.6%)	Pearson Chi^2 = 44.09
Secretor	93 (79.5%)	24 (20.5%)	P <0.001
Total	167	133	
Serum resistance			
Non-Secretor	34 (18.6%)	149 (81.4%)	Pearson Chi^2 = 0.21
Secretor	24 (20.5%)	93 (79.5%)	P = 0.899
Total	58	242	
Biofilm production			
Non- Secretor	46 (25.1%)	137 (74.9%)	Pearson Chi^2 =18.81
Secretor	58 (49.6%)	59 (50.4%)	P <0.001
Total	104	196	

Note: MSHA- Mannose sensitive hemagglutination; MRHA- Mannose resistant hemagglutination.

Table 6. Correlation of Secretor status with genotyping of virulence genes of *E coli* in UTI patients

Secretor Status	*hlyA* gene		Pearson Chi² P-value
	Absent N (%)	Present N (%)	
Non-Secretor	108 (59%)	75(41%)	Pearson Chi² = 39.23
Secretor	108 (92.3%)	9(7.7%)	P <0.001
Total	216	84	
		***cnf1* gene**	
Non- Secretor	93 (50.8%)	90 (49.2%)	Pearson Chi² = 17.74
Secretor	88 (75.2%)	29 (24.8%)	P <0.001
Total	181 (60.3%)	119(39.7%)	
		***iss* gene**	
Non- Secretor	134 (73.2%)	49 (26.8%)	Pearson Chi² = 6.23
Secretor	100 (85.5%)	17(14.5%)	P = 0.013
Total	234 (78%)	66(22%)	
		***fimH* gene**	
Non-Secretor	135 (73.8%)	48(26.2%)	Pearson Chi² = 8.8
Secretor	103 (88%)	14(12%)	P = 0.003
Total	238(79.3%)	62(20.7%)	
		***papA* gene**	
Non- Secretor	76 (41.5%)	107 (58.5%)	Pearson Chi² =52.25
Secretor	98 (83.8%)	19 (16.2%)	P <0.001
Total	174 (58%)	126(42%)	
		***iutA* gene**	
Non- Secretor	87 (47.5%)	94 (51.3%)	Pearson Chi² = 16.49
Secretor	83 (71%)	34(29%)	P = 0.0698
Total	170 (56.67%)	128(42.67%)	
		***csgA* gene**	
Non- Secretor	44 (24%)	139 (76%)	Pearson Chi² = 2.08
Secretor	37 (31.6%)	80(68.4%)	P = 0.149
Total	81 (27%)	219(73%)	

t-test. The ability of NLR to predict the varying duration of hospital stay was studied using one-way ANOVA.

RESULTS

A total of 2758 patients were recruited in the study, fulfilling inclusion and exclusion criteria and there was a significant male predominance (1711 men). Of the 2758 patients, 529 (19.2%) patients had significant bacteriuria, of whom 427 (80.7%) had infection with gram-negative bacilli of which *E. coli* was the predominant organism (56.7%).

Detailed patient demographics and clinical presentation of the patient is cited in our earlier publication, studying the antibiotic resistance in the same subset.[7] Patients in the study (n=300) had upper UTI due to E.coli, as defined by clinical symptoms and among them, 99 patients (33%) had AKI at initial presentation, as per KDIGO guidelines. At the end of 14 days follow-up, 60 patients (20%) had persistent AKI. Most of the patients were women (57.4%) in the age group of 26-65 years (64.7%). The majority of them were non-secretors (61%) and did not have diabetes mellitus (63%). 77.5% of the diabetic patients were non-secretors and this association was significant ($p < 0.000$). Patients with diabetes and non-secretors had a significant association with AKI at presentation as well as at 14 days follow-up ($p < 0.001$). (Table 2 and 4) Both diabetics and non-secretors had a higher average duration of hospitalization (> 5 days) (Table 3).

Virulence studies show higher expression of serum resistance 80.7% followed by biofilm production 65.3%, MRHA 44.3%, hemolysin 26%

Table 7. Association of Urine Microscopy Score with AKI at presentation, at 14 days and with duration of hospital stay

UM score	AKI at presentation		P-value	AKI at 14 days		Pearson Chi^2 P-value
	Absent	Present		Absent	Present	
1	201(91%)	20(9%)		217(98.2%)	4(1.8%)	
2	0(0%)	54(100%)	<0.001	23(42.6%)	31(57.4%)	<0.001
3	0(0%)	25(100%)		0(0%)	25(100%)	
Total	201(67%)	99(33%)		240(80%)	60(20%)	

UM Score	**Hospital stay in days**			Pearson Chi^2 P value
	Outpatient care	1-5	>5 days	
1	46(20.8%)	135(61.1%)	40(18.1%)	
2	8(14.8%)	13(24.1%)	33(61.1%)	<0.001
3	0(0%)	1(4%)	24(96%)	
Total	54(18%)	149(49.7%)	97(32.3%)	

Table 8. Receiver operating curve (ROC) values for Urine Microscopy Score

	Cut-off threshold (%)	Sensitivity (%)	Specificity
AKI at presentation	≥ 2	79.80	100
AKI at 7 days	≥ 2	88.24	98.14
AKI at 14 days follow up	≥ 2	93.33	90.42

Table 9. Association of Neutrophil lymphocyte ratio with AKI at presentation, at 14 days and with duration of hospital stay

AKI at presentation	Neutrophil lymphocyte ratio		P-value	AKI at 14 days	Neutrophil lymphocyte ratio		Two sample t-test P-value
	Mean	Std Dev			Mean	Std Dev	
Absent (201)	3.486	0.456		Absent (240)	3.570	0.531	p<0.001
Present (99)	4.407	0.778	0.000	Present (60)	4.668	0.735	
Total (300)				Total (300)			

Hospital stay in days	Neutrophil lymphocyte ratio		One-way Anova P value
	Mean	Standard Deviation	
O	3.011	0.031	p<0.001
1-5	3.50	0.335	
>5 days	4.66	0.468	

and MSHA 21%. (Table 4) Non-secretors were associated with most of the virulence genes (Table 6). Biofilm production was associated with virulence factors like Hemolysin production (p = 0.005) and fimbrial antigen hemagglutination (p = 0.003) except serum resistance. Virulence factors of UPEC viz., hemolysin production, type I and P fimbriae demonstrated by MSHA (p = 0.005) and MRHA and biofilm production had a significant association (P <0.001)with non-secretors along

Table 10. Receiver operating curve (ROC) values for neutrophil-lymphocyte ratio to predict AKI

AKI	Cut-off threshold	Sensitivity(%)	Specificity(%)
AKI at presentation	3.9	72.73	77.11
AKI at 7 days	4	77.65	77.67
AKI at 14 days follow up	4.3	78.33	88.33

with the presence of their corresponding genes. The only exception was the phenotypic expression of serum resistance, which did not correlate with non-secretors (p = 0.899) (Table 5).

UM score performed on the urine specimen at presentation, had a strong association with AKI at presentation and on the 14th day (p < 0.001). Similarly, UM score was useful in predicting patients with longer duration of hospital stay (Table 7). We found white blood casts (WBC casts) in UM in 68 patients (22.6%), but this had no correlation with AKI or the need for hospitalization.

NLR at presentation was useful in the identification of AKI at initial manifestation as well as on the 14th day and an increase in NLR was also significantly associated (p < 0.001) with a longer duration of hospital stay (Tables 9).

UM score and NLR were the parameters that predicted the AKI early and also assessed the severity of disease needing a longer duration of hospital stay. UM score of > 2 had the best threshold for sensitivity and specificity to predict AKI at presentation as well as on follow-up in receiver operating curve (ROC). ROC curve highlighted increasing thresholds (Tables 8 and 10).

DISCUSSION

Upper UTI is a treatable cause of AKI and delays in diagnosis lead to renal parenchymal damage. Our intention is to identify simple diagnostic tools to predict AKI and involvement of renal parenchyma in UTI patients. UM score and NLR were useful in the identification of AKI and assessed the severity of disease needing hospitalization and predicted persistence of AKI.

Secretor status is studied as an independent predisposing factor in several diseases. Most of the studies have assessed the FUT2 gene polymorphisms associated with the expression of secretors.[8,13] However, there are limited studies using phenotypic methods for the assessment of secretors. We used Pearson's Chi-square test for testing the hypothesis on the association of non-secretors with virulence factors of UPEC, as we could not find significant data in the literature on its association and the ensuing novelty. Even more, studies using the phenotypic method for assessment of secretor status were even rarer. We explored the association of virulence factors of UPEC by both phenotypic and genotypic methods. Our results were similar to that of Ziegler et al.,[14] and Stapleton et al.[15]

The presence of *iss* and *iutA* genes respectively play a part in the regulation of serum resistance and biofilm production among many other genes. There was a discordance in the statistical association with non-secretors between phenotypic expression of serum resistance, biofilm production with their corresponding *iss* and *iutA* genes. The presence of iss gene was significantly associated with non-secretors, while *iutA* gene was not. In addition, *csgA* gene, which was the most commonly found virulence gene in our study population of UPEC, did not correlate with non-secretors.

NLR assessed from hemogram taken at the time of initial presentation was found to be significantly useful in identifying AKI at presentation as well as in predicting the persistence of AKI during follow-up. In addition, NLR was also significantly useful in identifying patients who needed hospitalization and longer duration of in-hospital stay. The ROC curve indicated a threshold of 3.9 for diagnosis of AKI at initial presentation while when the NLR was extrapolated to predict AKI at 7 and 14 days, the threshold levels increased to 4 and 4.3. These values are similar to the findings in our previous study.[16]

NLR is associated with a range of diseases from exogenous steroid administration to cancer and this is a major limitation in its utility as a diagnostic or prognostic tool. Our patients did not

have any known malignancy, HIV infection or sepsis based on clinical evaluation, baseline ultrasound abdomen and laboratory workup. However, it is not possible to exclude all possible causes of elevated NLR and this is a limitation. In view of the popular use of NLR as a superior biomarker of severity in a wide spectrum of diseases, we have used the two-sample t-test to study the association of AKI in our patients at different time intervals and one way ANOVA to study the utility of NLR at presentation to predict the duration of hospital stay. Receiver operating characteristic curve was used to assess the best threshold of biomarkers, UM score and NLR with the best possible sensitivity and specificity in predicting AKI.

UM score >2 was found to be significantly useful in the identification of AKI at presentation as well as in the extrapolation to predict AKI during follow-up. In addition, this score was found to be significantly useful in identifying the need for as well as for the duration of hospitalization ($p < 0.000$). These findings were similar to that of Perazella et al.,[17] and Kana et al.[16]

None of the virulence factors were associated with the presence of AKI nor was there any association with the need for hospitalization or duration of admission. Our findings did not correlate with that of Wang et al.[18]

We found the association of virulence factors of UPEC with non-secretors ($p < 0.000$). This finding needs to be explored in a larger subset of patients with a longer duration of follow-up.

We attribute the elevated UM score due to acute tubular necrosis (ATN) in upper UTI. The pre-renal cause was not evaluated as patients with hypotension, liver disease and cardiac failure were excluded in our study. Fractional excretion of sodium (FeNA) was not assessed due to its limitations in the distinction of pre-renal from the renal cause of AKI.

To conclude, UM score and NLR are potentially useful biomarkers in identifying and predicting AKI as well as hospitalization in patients with upper UTI due to UPEC. Diabetics and non-secretors are found to be associated with AKI as well as the need and duration of hospitalization in this subset.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

None

DATA AVAILABILITY

The datasets generated during and analysed during the current study are available from the corresponding author on reasonable request.

ETHICS STATEMENT

This study was approved by the Institutional Ethics Committee, Indira Gandhi Government General Hospital and Postgraduate Institute, Puducherry vide Ref. No. GHEC/2016 dated 23rd December 2016.

INFORMED CONSENT

Written informed consent was obtained from the participants before enrolling in the study.

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