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EMERGING ROLE OF URINARY BIOMARKERS IN DETECTION OF UROTHELIAL BLADDER CARCINOMA IN SOUTH INDIAN POPULATION

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ABSTRACT

Urine cytology is used for screening of exfoliated bladder cells from voided urine but lacks sensitivity. This study aims to check the efficacy of 5-aminolevulinic acid (5- ALA) fluorescence cytology and establish a high sensitivity approach in detecting flat, *in-situ* and/or small lesions that are hardly visible under conventional cystoscopy. Intracellular PPIX allows red fluorescence detection. In this study, 5-ALA fluorescent cytology using urine was compared with conventional cytology in the diagnosis of bladder tumors. In this prospective study, we compared the sensitivity and specificity between conventional cytology, 5-ALA fluorescent cytology and FDA approved commercially available kits (NMP-22 and BTA). The percentage of Protoporphyrin IX facilitated by 5-ALA was amplified in cancer urothelial cells compared to normal urothelial cells. The sensitivity of conventional cytology and 5-ALA fluorescent cytology was 64% and 96% respectively, whereas the specificity was 92% and 98.67% respectively. In conclusion, 5-ALA induced fluorescent urine cytology demonstrated promising outcomes in the detection of bladder carcinoma cells. Furthermore, low grade and low stage tumor cells as well as flat lesions were also positively and accurately interpreted using 5-ALA fluorescent cytology.

Keywords: Bladder cancer, Non-invasive diagnosis, 5-Aminolevulinic Acid, Nuclear Matrix Protein, Bladder Tumor Antigen.

1. INTRODUCTION

Bladder cancer is one of the most common urological cancers. Bladder cancer is generally diagnosed by urethra-cystoscopy, which permits direct imagining of tumours and confirmation by biopsy and pathological analysis [1]. Nevertheless, Cystoscopy and voided urine cytology are effective diagnostic methods for investigation of superficial bladder cancer. Flexible cystoscopy being an invasive procedure has made cystoscopy more acceptable to patients [2]. Voided urine cytology remains the method of choice for the non-invasive detection of bladder cancer, yet whilst it has a specificity of 93%, its sensitivity is only 25-40%, especially for low-grade and T-stage tumours. Bladder cancer in males is 7th most common carcinoma as of 2020, and it has affected around 8.5 males and 3.2 females in every 100000 cases, around the globe. The bladder invariably comes in contact with the environment making it sensitive to environmental carcinogens and inflammation. Tobacco smoke containing aromatic amines, which when hydroxylated, lead to DNA damage [3].

Environmental carcinogens have been implicated in BC with a rising trend in the past decade due to increase in smoking habits. About 70-80% of the BC is diagnosed as non-muscle invasive (NMIBC) and 20-30% as muscle invasive (MIBC). An early diagnosis and detection of recurrence is of utmost importance as 10-30% of NMIBC progress to MIBC [4]. Cystoscopy and biopsy remain the gold-standard tests for the diagnosis of bladder cancer and enable the urologist to identify and resect all visible tumors. Cystoscopy has an overall sensitivity of 62-84%

and specificity of 43-98%, is cost-intensive, invasive and operator-dependent and has limited ability to pick small papillary tumors, low-grade urothelial neoplasms (LGUN) and *in-situ* tumours (CIS) [5]. The clinical spectrum at present can be divided into those with (i) non-muscle invasive bladder cancer, (ii) muscle-invasive bladder cancer, and (iii) metastatic disease. The muscle-invasive bladder cancer has a high recurrence rate of 50-70% as it reoccurs despite conservative measures such as transurethral of bladder tumor (TURBT) and intravesical therapy. However, screening individuals who are at high risk for bladder cancer, with a history of tobacco, occupational exposure, cyclophosphamide exposure, or pelvic radiation, may be helpful for the early detection of bladder cancer [6].

A wide range of alternative procedures and markers have been proposed and studied for the detection of recurrent bladder tumours. These include 5-Aminolevulinic Acid (5-ALA) cytology [7], nuclear matrix protein 22 (NMP-22) [8], BTA test [9] etc. The European Association of Urology in 2006 approved in using 5-Aminolevulinic Acid (5-ALA) for photodynamic diagnosis (PDD) of bladder tumor by cystoscopy as an effective procedure in detection and treatment for various cancer such as skin tumor, brain tumor, and oesophageal tumors. The principle behind 5-ALA fluorescence cytology is based on the metabolism of heme biosynthesis. 5-ALA being the precursor of heme metabolism will selectively get accumulated as protoporphyrin IX (PPIX) in tumor cells. The accumulation of ALA mediated PpIX was earlier reported to be 17 times higher than in normal mucosa. Moreover, ALA is impermeable through lipid bilayers and the metabolism of heme occurs both in cytosol and mitochondria, hence the efficacy of ALA dependent PDD is limited by cellular uptake of ALA and additional accumulation of photosensitizer PpIX [10, 11]. This occurrence is due to the numerous facts such as increased uptake of ALA in tumor cells, mitochondrial properties, and modification of enzyme activity in enzymes such as porphobilinogen deaminase and ferrochelatase activity and storage of PPIX in malignant cells. The sensitivity of PDD with 5-ALA for Transurethral resection of bladder tumor (TURBT) is higher compared to routine white light cystoscopy [12].

However, cystoscopy with 5-ALA has a higher false-positive rate than white light cystoscopy. In contrast, Use of Aminolevulinic Acid provides great benefits compared to conventional cytological procedures. Previous reports have investigated the feasibility and usefulness of urine base tests taking advantage of ALA-fluorescence [13]. Furthermore, the detection modalities are based on fluorescence cytology, fluorescence spectrophotometry, and flow cytometry. However, detection modalities such as spectrophotometry and flow cytometry require cumbersome procedures and costly equipment. In the search for a highly specific, sensitive, and objective method for detection for urothelial tumor cells, we have utilized the ability of fluorescence using 5-ALA for the diagnosis of urothelial tumor cells [14].

For quantitative analysis, FDA approved commercial test were used which follows the principle of enzyme linked immunoassay. First test include NMP-22, it is a nuclear mitotic apparatus protein is present in the nuclear matrix of all cell types and located in the mitotic spindle during mitosis is involved in the proper supply of chromatin to daughter. In bladder cancer, NMP-22 is twice as sensitive as cytology in detecting early T-stage cancers, and up to 90% sensitive and 99% specific [15]. Similarly, BTA has been identified as a human complement factor H related protein (hCFHrp), which is produced by bladder tumour cells in cell cultures and not by any other epithelial cell lines. BTA is released into the urine of patients with bladder cancer as the tumour invades the stroma. Initial reports indicated that BTA test had higher sensitivity and lower specificity than cytology [16]. The present study aimed to detect urothelial bladder carcinoma using 5-ALA and its comparison with conventional cytology, NMP-22 and BTA TRAK quantitative test to estimate activity in the urine of the patients with bladder cancer in a trial to assess their value in the detection of the tumours and to find a reliable non-invasive technique for the diagnosis of cancer bladder. Sensitivity and specificity of these tumour markers was compared to conventional cytology in bladder cancer.

2. MATERIAL AND METHODS

2.1. Patients

The study was conducted between September 2019 and February 2022 at the Urology clinic in a tertiary care centre of South India. The study was reviewed and approved by the institutional ethics committee (KAHER/EC/20-21/001/05). A total of 250 patients with 128 Bladder carcinoma (Cases) and 122 non-cancerous (Controls) were studies. Patients with signs or symptoms suggestive of bladder cancer and patients followed up for a past history of treated bladder cancer were included in the study. Furthermore, patients with a history of renal involvement (e.g. calculi, nephritis or

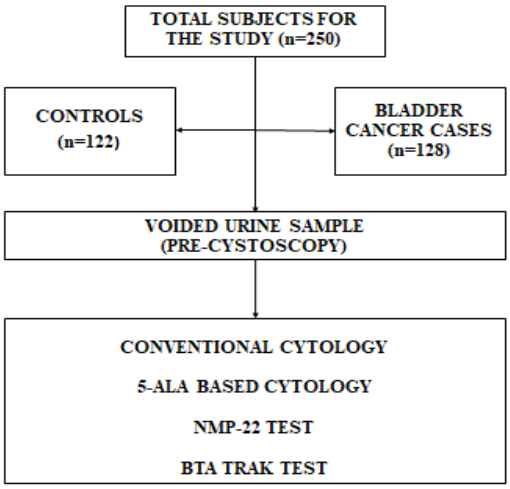
renal cancer); as these conditions may affect bladder cancer antigen detection; recent trauma of the bladder (e.g. cystoscopy, vesical washing, biopsy, or surgery); radiotherapy in the last 3 months; systemic chemotherapy in the last month; gross haematuria in the sample and urine infection formed the control group.

2.2. Collection of voided urine sample

Pre-operative urine sample of 150 cc was collected and samples were divided into four different groups, and the following procedures were performed within 1 h for pathological examination. We separated these samples for the conventional cytology [18], ALA-induced fluorescent cytology [10], NMP-22 and BTA-TRAK (Fig. 1).

2.3. Evaluation of ALA based cytology

Two ml of Minimum Essential Medium (MEM 11095080, GibcoTM, Thermo Fisher Scientific, Waltham, MA, USA) was added to the urine sediment. 5-ALA (5-Aminolevulinic Acid Hydrochloride, Sigma-Aldrich, ©Merck KGaA, Darmstadt, Germany, 2020) was added to the cell suspension to 200µg/ml and incubated for 2 hours at 37°C in a light-shielded incubator. Then, centrifuged the mixture again (1500 rpm for 5 min). The cell suspension was loaded on a slide and was observed using a fluorescence microscope (Nikon ECLIPSE Ni; Nikon Corporation, Tokyo, Japan) loaded with a 400-440 nm bandpass filter for excitation and a 610 nm long-pass filter for absorption, and the presence of fluorescent and reddish urothelial epithelial cells was diagnosed. The brightness and contrast of the fluorescence microscope were set to the recommended default settings for fluorescent urine cytology (Fig. 2).



Total subjects enrolled in the study were divided into two sub-groups i.e. cases and controls. Voided urine samples were subjected to urinary test such as conventional cytology, 5-ALA based cytology, NMP-22 test and BTA TRAK test. ALA- Aminolevulinic acid; NMP22-Nuclear matrix protein; BTA-Bladder tumour antigen.

Fig. 1: Samples for different tests

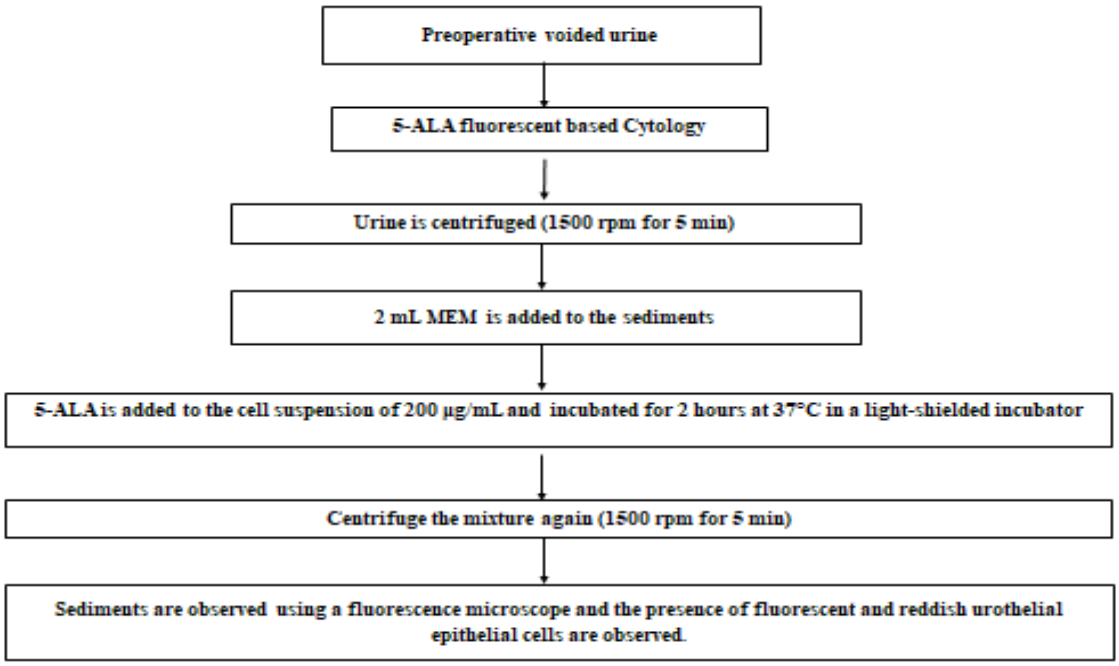


Fig. 2: The protocol of the 5-Aminolevulinic acid fluorescence detection assays. MEM- Minimum essential media

The cells with faint red or pink colour and histogram depicting peaks with low intensity versus frequency were considered as negative for malignancy. Cells showing bright red fluorescence against black background and its histogram depicting peaks (>200, 10K) of high intensity versus frequency were considered malignant (Fig. 3 & 4).

Cells were incubated with 5-ALA for 1 hr. The sediments were observed under fluorescence microscope. For Fig. (a & c), cells showed light red or pink colour against black background suggesting benign urothelial cells. (200X). For Fig. (b & d) the histogram showed intensity (x-axis) vs frequency (y-axis) to be minimum or normal.

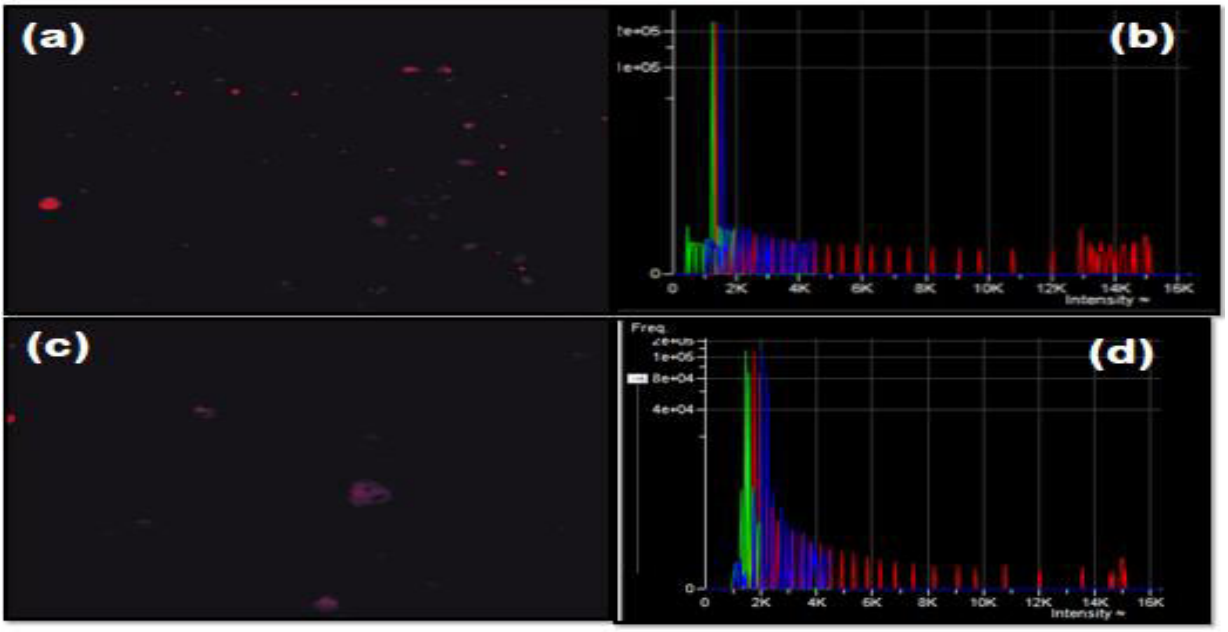


Fig. 3: Effect of 5-ALA on voided urine

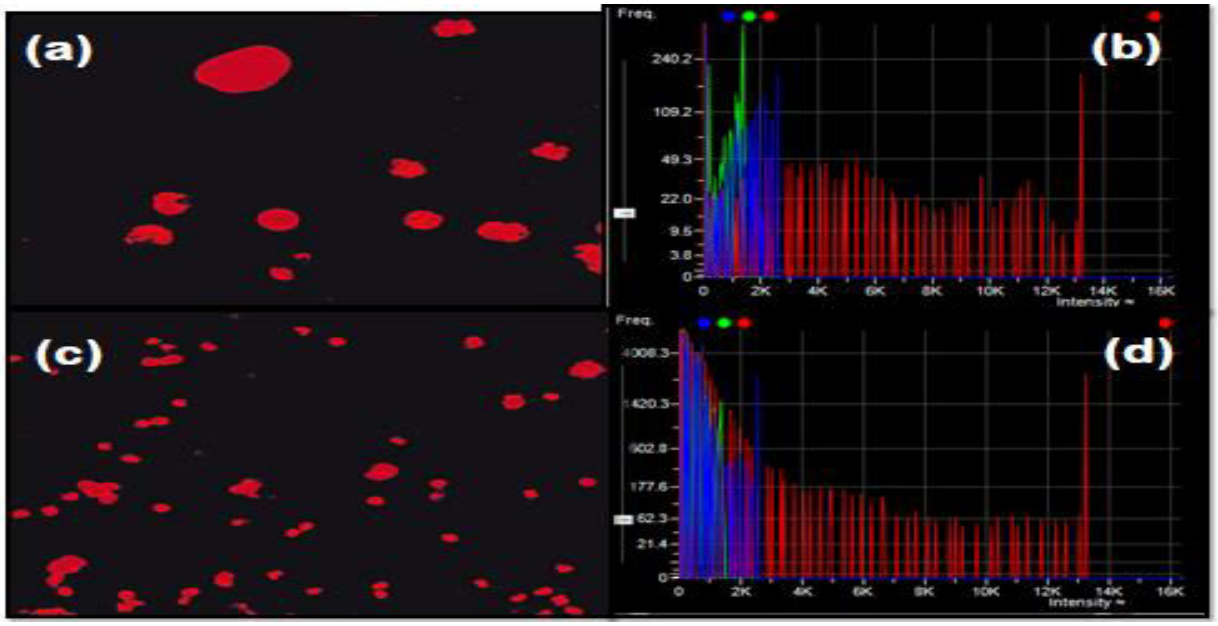


Fig. 4: Effect of 5-ALA on voided urine

Cells were incubated with 5-ALA for 1 hr. The sediments were observed under fluorescence microscope. For Fig. (a & c), cells showed bright red or dark red colour against black background suggesting

malignant urothelial cells. (200X). For Fig. (b & d) the histogram showed intensity (x-axis) vs frequency (y-axis) to be increased.

2.4. Evaluation with commercially available KIT methods

The cell free urine partwas used to evaluate NMP-22 and BTA TRAKby an enzyme immunoassay (EIA) employing two monoclonal antibodies that were specific for the NMP-22 antigen moiety of nuclear mitotic apparatus protein, were used to determine NMP-22 levels in stabilized voided urine (MyBiosource NMP22® test kit, South California, USA). Assay sensitivity, i.e. the lowest concentration of NMP22 antigen that can be measured reliably, is 0.156ng/ml.; within- and between-run coefficients of variation were 4.9 and 9.5%, (Detection Range: 0.156 ng/ml-40 ng/ml.) according to manufacturer. The BTA TRAK assay (MyBiosource NMP22® test kit, South California, USA) is an immunoenzymatic assay (IEMA) utilizing monoclonal antibodies to bind specifically to bladder tumor antigen in urine. The lowest concentration of BTA detectable was estimated to be 19ng/mL. Assay reproducibility as indicated by kit purchaser yielded 5 and 10% variation in intra- and extra-series tests, respectively (Detection Range: 0.31-20ng/mL).

2.5. Analysis of results

Average o fthe duplicate readings for each standard and samples was calculated, then subtracted the average zero standard optical density. a four-parameter logistic curve on log-log graph paper was plotted, with standard concentration on the x-axis and OD values on the y-axis. If the samples have been diluted, the concentration calculated from the standard curve must be multiplied by the dilution factor. If the OD of the sample surpasses the upper limit of the standard curve, a re-test must be done with an appropriate dilution. The actual concentration is the calculated concentration multiplied by the dilution factor.

2.6. Statistical Analysis

Data were compared using the Wilcoxon test or Chi-square test. Differences were considered statistically significant when $p<0.05$. Statistical analyses were performed using SPSS 22.0.

3. RESULTS

Table 1 shows the patients' clinico-pathological characteristics. The group with UC was older (median age 54 years old) than the non-cancer group (median age 53 years old)($p < 0.01$). The UC group included 128 patients with BT. The non-cancer group included 58 patients with BPH, 45 with urinary stones, 13 with UTI, and 6 with other with urological diagnosis. In the cancer group, 115 patients had cancer of pT1 or less (40, 18, and 57 patients with pTa, pTis, and pT1, respectively), 13 had cancer of pT2 or more, 57 had a low-grade tumor, and 70 had a high-grade tumour (Table 2).

The comparison of conventional cytology, 5-ALA cytology, NMP-22 test and BTA TRAK testwith respect to sensitivity in all specimens are presented in (Table 2). In subgroup analysiswe found that 5-ALA-induced fluorescent cytology tended to be more sensitive than conventional cytology and other kit based biomarkers regardless of patient age (classified by the median age of 54 years old) and sex. The sensitivity was significantly higher for 5-ALA-induced fluorescent cytology (91%) versus that for conventional cytology (55%), NMP-22 test (60%) and BTA TRAK test (64%). Similar trends were seen in both low grade (82%) and high grade (97%) tumours for 5-ALA cytology. Out of 115 BT cases pT1 104 cases were positive for 5-ALA cytology (90%) and for pT2 or more the sensitivity was significant higher compared to other biomarkers (92%, <0.0001).

However, in (Table 3), the specificity (98.2% vs. 95.6% vs 89.21 vs 92.10, $p = 1.0$) and positive predictive value (97.22% vs. 95.08%, vs 85.56 vs 89.13$p = 0.57$) were equivalently high between 5-ALA-induced fluorescent cytology and other biomarkers. In conventional cytology, false positives were found in two patients: one with Bladder prostate hyperplasia (BPH), and one with urinary tract infection (UTI). In 5-ALA-induced fluorescent cytology, false positives were found in six patients: two with BPH, two with UTI, and two with urinary stones. In NMP-22 test, false positives were found in thirteen patients: six with BPH, three with UTI, two with urinary stones and two in radiological cystitis. Similarly, for BTA TRAK test ten cases shown false positive results.

In cases judged as false positive by the conventional cytology or 5-ALA-induced fluorescent cytology, transurethral bladder random biopsy, selective upper tract urine cytology, and contrast-enhanced CT were performed to examine the urinary tract tumor. There were no findings to indicate UC either by additional imaging or pathological examinations, or in the

subsequent follow-up of all patients, urine cytology was re-examined every 3 months for over 1 year and confirmed to be negative. These results suggested that ALA-induced fluorescent cytology may be a superior tool to conventional cytology in clinical practice. When compared among all test, 5-ALA induced fluorescence cytology showed highest Cohen's Kappa (K) of 0.8745 complying to near perfect strength of agreement.For this study, therespective sensitivity and specificity of rangedfrom 40-70% for the conventional cytology, 80-95% for the 5-ALA based cytology, 40-60% for the nuclear matrix protein (NMP) 22 test, and 50-65% for the bladder tumor-associated antigen (BTA) test. The time taken for the examination of one urine specimen is about 1-2 days for conventional cytology, 180 min for 5-ALA based cytology, 300 min for NMP 22 and 240 min for BTA. In addition, the cost of one test is INR 350-400 for conventional cytology, INR 250 for ALA-induced fluorescent cytology andINR 1000-1500 for both NMP 22 and BTA TRAKas described above, 5-ALA-induced fluorescent cytology showed high sensitivities within a short period of time and at much lower cost compared with these other urinary biomarkers (Table 3).

Table 1: Characteristics of Study Subjects

Parameters	Bladder Cancer (%) (n=128)	Controls (%) (n=122)
Mean Age (Range, Years)	54.14 (29-85)	53.34 (24-90)
Male: Female	(114:14)	(105:17)
Comorbidity		
Hypertension	32 (25)	16 (13)
Type- I DM	2 (2)	1 (1)
Type- II DM	24 (19)	15 (12)
Chronic Kidney Disease	11 (9)	3 (2)
Ischemic Heart Disease	8 (6)	4 (3)
Thyroid Disorder	2 (2)	0
Multiple comorbidity	11 (9)	7 (6)
None	38 (30)	76 (62)
Diagnosis		
Bladder Carcinoma	128 (100)	0
BPH	-	58 (48)
Urolithiasis	-	45 (37)
Infection	-	13 (11)
Others	-	6 (5)
Occupational Exposure		
YES	79 (62)	47 (39)
NO	49 (38)	75 (61)
Family History of Any Cancer		
YES	21 (16)	8 (7)
NO	107 (84)	114 (93)
History of Urological Infection		
YES	60 (47)	36 (30)
NO	68 (53)	86 (70)

Table 2: Diagnostic Sensitivity Comparison Between Urinary Biomarkers

Diagnostic Test	Bladder Cancer							Total (%)	Control (%)
	Tumour Grade (%)		Pathological T-Stage (%)						
	Low	High	Ta	Tis	T1	T2	T2>		
	57	71	40	18	57	8	5	128	122
Conventional Cytology	18 (32)	52 (73)	18(45)	8(44)	37(65)	4(50)	3(60)	70(55)	2(2)
5-ALA Cytology	47 (82)	69 (97)	38 (95)	12 (67)	54 (95)	7 (88)	5 (100)	116 (91)	6 (5)
NMP-22 TEST	26 (46)	53 (75)	19 (48)	12 (67)	35 (68)	6 (75)	5 (100)	77 (60)	13 (11)
BTA TRAK TEST	27 (47)	55 (77)	25 (63)	13 (72)	35 (61)	5 (63)	4 (80)	82 (64)	10 (8)

Table 3: Sensitivity, Specificity, Inspection Time, and Cost of Urinary Biomarkers

Variables	Conventional Cytology	5-ALA Cytology	NMP-22 TEST	BTA TRAK TEST
Sensitivity	55%	91%	60%	64%
Specificity	98%	95%	89%	92%
Positive Predictive Value (PPV)	97%	95%	86%	89%
Negative Predictive Value (NPV)	67%	91%	68%	71%
Accuracy	76%	93%	75%	78%
Cohen's KAPPA (K)	0.4561	0.8745	0.4214	0.4917
Inspection Time	1-2 days	180 min	300 min	240 min
Cost (INR)	350-400	250	1000-1500	1000-1500

4. DISCUSSION

Although numerous new urine based test for the detection of bladder cancer are available. Invasive approach like cystoscopy is the number one diagnostic modality for the diagnosis new or recurrent bladder carcinoma.Non-invasive methods likevoided urinary cytology remains the most established non-invasive method for detecting bladder cancer. However, this method has several drawbacks including low sensitivity, low-cost effectiveness, a lack of inter observer variability, and technical instability [17]. Moreover, its sensitivity is low: between 11% and 76% in various studies [18]. Several factors affect the sensitivity of cytology, including specimen quality, number of exfoliated cells and pathologist expertise. The overall low sensitivity of cytology is due to its low sensitivity in detecting low-grade bladder tumours. Non-invasive urine markers can offer an alternative to the standard mode of detecting bladder cancer or they can be used as an adjunct to cystoscopy [19]. In addition, cytological evaluation is subject to the pathologist's experience. The limitations of both techniques led to the development of several urine-bound tests for the early diagnosis of bladder cancer [20]. For this purpose, new bladder cancer markers have recently been introduced into clinical practice.

Currently, 5-ALA is approved as a photosensitizer of photo dynamic diagnosis (PDD) for carcinoma around the world. For example, ALA as an optical imaging medicine was approved to enhance intraoperative detection of malignant glioma and also to detect bladder cancer [21]. A recent report on 5-ALA staining of urine specimens in an extracorporeal exposure showed PDD sensitivity to be effective compared with conventional cytology in BT (82% vs. 49%, respectively), particularly in low-grade and low-stage tumors, and to have comparable specificity (80% vs. 100%, respectively) [14]. However, they studied a small number of patients (61 with BT only), and there were some limitations in that conventional cytology for low-grade BT had very low sensitivity (18%), and the details of the non-cancer group were not described. For NMP-22 test, Research has found that patients with bladder cancer may have urinary levels of NMP22 that are 25-fold greater than levels in healthy subjects [22]. Similarly, BTA TRAK assay have so far been promising, with the finding of an overall sensitivity of about 70%. Conversely, urine cytology sensitivity is much lower in the same authors' experience, never exceeding 44% [23].

The present study included more patients (n = 250). Our results indicated that the sensitivity of 5-ALA-induced fluorescent cytology was significantly higher than that of conventional cytology, NMP-22 test and BTA TRAK test, especially for tumours of pTa stage and low-grade tumors. In Low and High Grades and Ta, T1 stages, the sensitivity between 5-ALA cytology and the other two tests is more evident, not only in our series (sensitivity 95% in Ta and 95% in T1; 82% in LG and 97% in HG with 5-ALA cytology) but also in the studies by Yamamichi et al.[7]In all the three tests, a gradual improvement in the sensitivity with increasing grade and stage was noted, as similar to other comparison studies [24, 25].

Some reports indicated that the false-positive findings of 5-ALA cytology can be induced by several factors such as infection, inflammation, hyperplasia, and inexperience [26]. There were only six cases of false positives by 5-ALA cytology in the present study, probably because of inflammation associated with infection and calculi. There are couple of possible reasons for this, the first cellular components in the voided urine specimen, and the second being that urinary cellular components together with the cancer cells expired out over the passage of time from sample pooling to pathological examination. In this case, those

most cancers cells can notably lose their mitochondrial metabolic action, and therefore, 5-ALA cannot be metabolized. To prevent the death of these cancer cells in the present study, we treated the extracorporeal ALA culture within 1 h of collecting the urine samples.

There are some limitations in this study. Our study is a prospective study of patients from a single institution, and thus, the present results need to be validated in other cohorts to definitively identify the high diagnostic efficacy of 5-ALA-induced fluorescent urine cytology for UC. With an objective to find reliable, non-invasive technique for the diagnosis of bladder cancer, all the voided urine samples of patients were evaluated for NMP-22, BTA-TRAK, conventional cytology, and 5-ALA cytology. When the clinical history and examination is suspicious/suggestive of bladder cancer, a non-invasive diagnostic procedure may lead to an immediate diagnosis surpassing the need of cystoscopy and biopsy. In the follow-up of superficial bladder cancer patients, the ability of a urine test to detect a tumor recurrence could be a useful tool for the selection of cases to be submitted for control cystoscopies [25].

There is an unmet need for a simple reliable test that can screen a patient, serve as a diagnostic test, reduce the number of unnecessary biopsies creating less morbidity, decrease the healthcare costs and play a vital role in the determination of the effectiveness of therapeutic interventions. An ideal biomarker to detect bladder carcinoma should be (i) cost-effective, tangible, fast to process, and easy to interpret, with high sensitivity and specificity (ii) reduce the need for frequent invasive procedures; (iii) exclude recurrence; (iv) detect progression towards invasive disease; (v) predict effective treatment response [28, 29]. From our study we have clearly seen that the sensitivities and NPVs of 5-ALA induced cytology is higher than the conventional methods such as urine cytology, detection of BTA-TRAK and NMP-22. Our results are comparable with those obtained by other investigators. Our study with 5-ALA cytology is simple, reliable, feasible and efficient way to diagnose BC and can easily be done in most of the centres dealing with patients of BC.

5. CONCLUSION

In conclusion, 5-ALA based fluorescent cytology in the detection of bladder cancer in voided urine sample represents a set of novel diagnostic assays that can be employed for an early and accurate detection of BC, with highest sensitivity as compared to other routine methods. These can also efficiently detect low-grade urothelial tumors by showing positivity in different filters. Our study was a single-centred assessment and needs to be validated in other cohorts & multiple centres. We suggest validation and inscription of these markers in urine as independent diagnostic tests or in a panel to be recommended as these may play a vital role in early detection and diagnosis of bladder cancer.

Conflicts of Interest

The authors declared conflict of interest as None.

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