5-ALA-Induced Fluorescent Urine Cytology in Comparison with Conventional Cytology, BTA-TRAK, and NMP-22 Tests in the Diagnosis of Bladder Cancer

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Abstract

Introduction To assess the sensitivity fluorescent urine cytology induced by 5-aminolevulinic acid (5-ALA) in the diagnosis of bladder cancer and to compare the sensitivity and specificity with currently available markers approved by the United States Food and Drug Administration (FDA), bladder tumour antigen (BTA-TRAK, Bard Diagnostic Sciences, Redmond, WA, United States) assay based on enzyme-linked immunosorbent assay (ELISA), nuclear matrix protein 22 (NMP-22), and conventional cytology.

Materials and Methods Age- and gender-matched patients $\geq$ 18 years of age admitted with imaging-confirmed bladder cancer and non-malignant conditions formed the cases and controls respectively. A freshly-collected voided urine sample was divided into four parts, with each part used to perform: a) conventional cytology with Papanicolaou staining; b) the BTA-TRAK assay; c) the NMP-22 assay; and d) 5-ALA-induced fluorescent urine cytology. The resected bladder specimen was sent for histopathological examination.

Results In low-grade bladder cancers, the sensitivity of 5-ALA fluorescent cytology was of 88.02%, which was significantly higher than conventional cytology ($p < 0.0001$), the NMP-22 assay ($p < 0.0035$), and the BTA-TRAK assay ($p < 0.0007$). The sensitivity of 5-ALA fluorescent cytology was significantly higher in high-grade lesions when compared to conventional cytology ($p < 0.0005$) and the BTA-TRAK assay ($p < 0.039$).

Conclusions Fluorescent urine cytology induced by 5-aminolevulinic acid is a highly-sensitive test in the diagnosis of bladder cancer, and its sensitivity rates are significantly superior to those of conventional cytology, the NMP-22 assay, and the BTA-TRAK assay.

Keywords
- bladder cancer
- cytology
- urothelial carcinoma
- 5-aminolevulinic acid
- sensitivity
- specificity

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Introduction

Bladder cancer is a global disease, with 573,278 new cases and 212,536 deaths reported in 2020 worldwide.[1] The incidence and prevalence of bladder cancer increases with age. Nearly three-quarters of bladder cancer cases occur in males, who present a higher incidence rate (9.0 per 100 thousand) compared with women (2.2 per 100 thousand).[2] Bladder cancer is reported to be the most expensive cancer to treat per patient over a patient's lifetime.[3] Bladder cancer health care costs vary widely among countries and depending on the stage of the disease; moreover, these variations among countries and among patients in the same country could be attributed to regional variations in the management of bladder cancer, particularly in cases of non-muscle-invasive disease.[4]

Bladder cancer is rarely discovered incidentally, with painless gross hematuria being the presenting symptom in 85% of the newly-diagnosed patient, and microscopic hematuria is present in nearly all patients.[5,6] The patients can also present irritative voiding symptoms (such as frequency, urgency), which are also known to be signs of bladder cancer, particularly carcinoma in situ (CIS).[2] A patient with gross hematuria and suspected to have bladder cancer needs to undergo a full evaluation that includes a focused history and physical examination, cystoscopy, upper-tract imaging, and a urine culture. Patients presenting with gross hematuria should undergo upper-tract imaging with a multiphase computed tomography (CT) scan with delayed phase images. The 2012 American Urological Association (AUA) guidelines suggest a CT urogram (CTU) for the evaluation of asymptomatic microhematuria (AMH) as well; however, the CTU could be safely and easily replaced with a renal and bladder ultrasound in patients with AMH.[7] It would also be reasonable to obtain a urine cytology and/or urine markers in all patients with gross hematuria.[2]

Even today, the gold-standard tests for the diagnosis of bladder cancer are still cystoscopy and biopsy. Flexible office-based cystoscopy as well as rigid endoscopy are reliable for the diagnosis of bladder cancer.[8] Currently, white-light cystoscopy (WLC) is commonly used and remains the standard of care for the diagnosis of a bladder tumor, as it enables the urologist to map and resect all visible tumors. Although WLC has excellent sensitivity and specificity for the diagnosis of large papillary tumors, it is less reliable in the diagnosis of small papillary tumors and CIS. In such cases, porphyrin-induced fluorescent cystoscopy would be helpful, as it uses photoactive porphyrins, such as hexaminolevulinate (HAL), to emit red fluorescence under blue-wavelength light (360–450 nm).[9]

Urine cytology is a standard diagnostic test used to aid in the diagnosis of bladder cancer. The current sensitivity and specificity rates of urine cytology in the detection of bladder cancer range from 31 to 62% and 94 to 100% respectively.[10,11] Urine-based biomarkers are being developed as an adjunct to the standard methods used to diagnose and monitor bladder cancer. Non-invasive testing with sensitivity rates higher than those of urine cytology has been proposed as a desirable alternative to cystoscopy, which is costly and uncomfortable. Several urine-based biomarkers have been developed with higher sensitivity compared with that if urine cytology, although most still lack acceptable specificity rates.

Nuclear matrix protein 22 (NMP-22) is a test approved by the United States Food and Drug Administration (FDA) for use in bladder cancer surveillance. In a meta-analysis of 19 studies,[2] the sensitivity of quantitative NMP-22 was of 0.69 (95% confidence interval [95%CI]: 0.62–0.75), and the specificity was of 0.77 (95%CI: 0.70–0.83 k). The bladder tumor antigen (BTA-TRAK, Bard Diagnostic Sciences, Redmond, WA, United States) is an FDA-approved assay used in the diagnosis and follow-up of bladder cancer; it is a qualitative dipstick-based point-of-care test with a sensitivity of 0.64 (95%CI: 0.58–0.69) across 22 studies, and a specificity of 0.77 (95%CI 0.73–0.81). In addition, a quantitative BTA-TRAK based on enzyme-linked immunosorbent assay (ELISA) has also been developed, with a reported sensitivity of 0.65 (95%CI: 0.54–0.75) and a specificity of 0.74 (95%CI: 0.64–0.82 U/ml).[12]

Fluorescent urine cytology induced by 5-aminolevulinic acid (5-ALA) has also been used in the diagnosis of bladder tumours,[13,14] and is reported to be more sensitive than other non-invasive tests.[15] The objective of the present study was to assess and validate the use of 5-ALA-induced fluorescent urine cytology in the diagnosis of bladder cancer using voided urine samples and to compare the sensitivity and specificity with those of currently-available FDA-approved markers, ELISA-based BTA-TRAK, NMP-22, and conventional cytology.

Materials and Methods

The present prospective study was conducted with the approval of the institutional Ethics in Research Committee. All patients $\geq$ 18 years of age with an bladder cancer confirmed through imaging (ultrasonography/computed tomography) formed the case group. Age- and gender-matched patients $\geq$ 18 years of age admitted with non-malignant conditions, such as benign prostatic hyperplasia (BPH), urinary stone, urinary tract infection (UTI), ureteropelvic junction (UPJ) obstruction, and ultrasonography-confirmed cystitis formed the controls. All patients were asked to provide a freshly-voided urine sample. The collected sample was divided into four parts, with each part used to perform: a) conventional cytology with Papanicolaou staining; b) the BTA-TRAK assay; c) the NMP-22 assay; and d) 5-ALA induced fluorescent urine cytology.

Conventional Cytology[16]

The ThinPrep (Cytyc Corporation, Boxborough, MA, United States) technique was used to prepare slides from the voided urine samples. Most erythrocytes and leukocytes were removed by applying gentle negative pressure to assist filtration. This usually deformed these cells as they passed through the filter. A single layer of cells (monolayer) was obtained by gently pressing the filter against a pair of glass slides. The sample on the slide was fixed and the cell preparations were subsequently stained by the Papanicolaou method.

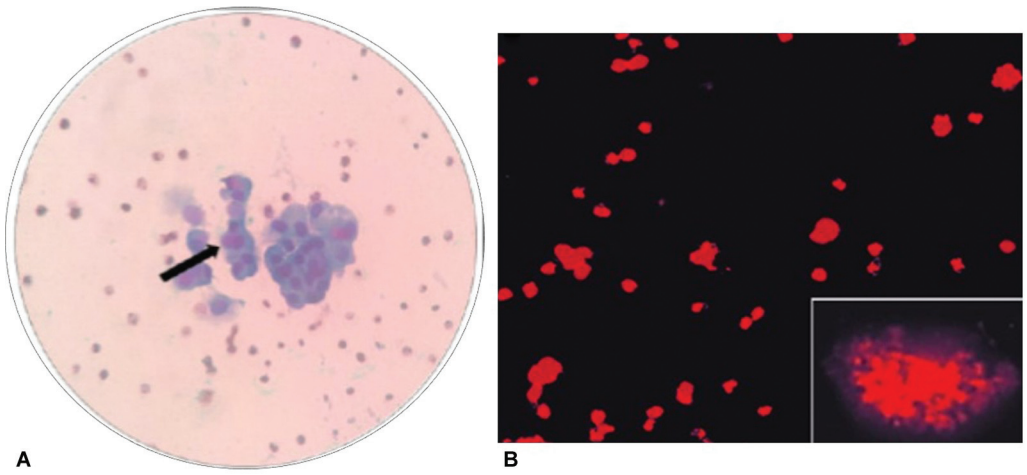

Fig. 1 (A) Conventional cytology stained with Papanicolaou staining showing clusters of large round pleomorphic urothelial cells with nuclear atypia. (B) 5-aminolevulinic acid (5-ALA)-induced fluorescent cytology showing cells with a dark red color against a black background, suggesting malignant urothelial cells.

Urine Samples and Treatment with 5-ALA[13]

The urine sample was centrifuged at 1,500 rpm for 5 minutes, and the supernatant was decanted. The pellet was suspended in a minimum essential medium (MEM) with 5-ALA hydrochloride (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany), and the concentration was adjusted to 200 µg/mL. Then, the suspension was stored in the dark at 37°C for 2 hours. After that, the sample was once more centrifuged again at 1,500 rpm for 5 minutes, and the pellet was resuspended in MEM. Finally, the urine sample was tested for protoporphyrin IX (PpIX) fluorescence using a fluorescent microscope (Nikon ECLIPSE Ni; Nikon Corporation, Tokyo, Japan) at appropriate settings (excitation wavelength of 405 nm and emissions wavelength of 600–650 nm).

NMP22 ELISA Assay[13]

The NMP22 ELISA assay employed the competitive enzyme immunoassay technique. The standards and urine samples were added to the microfilter with antibodies specific to NMP-22 and horseradish peroxidase (HRP) conjugated with a goat-anti-mouse antibody. A substrate solution was added to the wells, and the color developed in the sample is observed. The intensity of the color was measured.

BTA-TRAK Assay[13]

The desired number of coated wells in the plate holder was added with 10 uL of standard or urine samples to the appropriate wells. To this 100 uL of HRP conjugate was added and incubated; 90 uL of substrate reagent was added and incubated and, lastly, 50 uL of stop solution was added and the slides were read at 450 nm wavelength.

Evaluation

Conventional cytology was evaluated by one pathologist, and the NMP-22 assay, the BTA-TRAK assay, and 5-ALA-induced fluorescent cytology were evaluated by another pathologist using the same urine sample. The conventional urine cytology was considered either negative or positive for malignant cells based on The Paris System for Reporting Urinary Cytology" (►**Fig. 1A**). The 5-ALA-induced fluorescent cytology showing no red light was classified as negative, and the cells showing intense red were classified as positive for malignant. The final reading was confirmed by two pathologists (►**Fig. 1B**).

Comparison with Histopathology

All patients with imaging-confirmed tumors underwent cystoscopy/biopsy/transurethral resection of bladder tumor (TURBT). The surgical specimens were sent for histopathological examination and reported by the same pathologist. The histopathology reports were compared to the results of conventional cytology, the NMP-22 assays, the BTA-TRAK assays, and 5-ALA-induced fluorescent cytology (►**Fig. 2**).

Statistical Analysis

Data was analyzed using the Wilcoxon test or the Chi-squared test. Differences were considered statistically significant when $p < 0.05$. The statistical analyses were performed using the IBM SPSS Statistics for Windows (IBM Corp., Armonk, NY, United States) software, version 22.0.

Results

During the study period, a total of 150 patients with imaging-confirmed bladder cancer were included in the case group. During the same period, another group of 150 patients who were age- and gender-matched and admitted to the hospital with either lower urinary tract symptoms (LUTS) or other urological conditions were included in the study as controls. The mean age and gender distribution are as shown in ►**Table 1**. In total, 143 patients in the case group underwent cystoscopy followed by TURBT, and 7 patients were only submitted to biopsy. The histopathological examination of the surgical specimens confirmed transitional-cell bladder cancer in all 150 patients. The results of conventional cytology, the NMP-22 assays, the BTA-TRAK assays and 5-ALA-

Area Under the Curve					
Test Result Variable(s)	Area	Std. Error[a]	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval	
				Lower Bound	Upper Bound
NMP22	.867	.054	.000	.762	.972
BTA	.851	.052	.000	.750	.952
The test result variable(s): NMP22, BTA has at least one tie between the positive actual state group and the negative actual state group. Statistics may be biased.					
a. Under the nonparametric assumption					

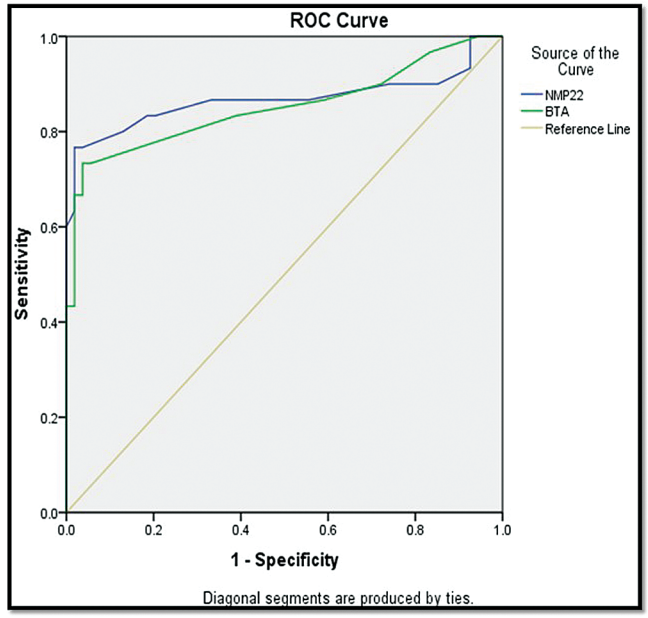

Fig. 2 Receiver operating characteristic (ROC) curve for the bladder tumor antigen (BTA-TRAK) and nuclear matrix protein 22 (NMP-22) assays.

Table 1 Age and gender distribution of the study sample

Demographics	Patients with imaging-confirmed bladder cancer (n = 150)	Age- and gender-matched controls (n = 150)	*p*-value
Mean age (years)	58.39	57.10	0.735
Male gender: n (%)	108 (72%)	110 (74%)	0.795
Female gender: n (%)	42 (28%)	40 (26%)	
Clinical diagnosis: n (%)			
Benign prostatic hyperplasia	–	54 (36%)	
Urolithiasis	–	36 (24%)	
Voiding dysfunction	–	42 (28%)	
Others	–	18 (12%)	
Bladder cancer	150	Nil	

induced fluorescent cytology of the cases and controls are shown in ►**Table 2**.

The sensitivity of 5-ALA fluorescent cytology was significantly superior to that of the other tests, with the *p*-value being highly significant. The specificity of all the tests was similar in the diagnosis of bladder cancer. The specificity of the various tests among the cases was higher in patients with high-grade lesions (►**Table 3**) when compared with those with low-grade lesions. In low-grade bladder cancers, the sensitivity of 5-ALA fluorescent cytology was of 88.02%, which was significantly higher than the rates for conventional cytology ($p < 0.0001$), the NMP-22 assay ($p < 0.0035$), and BTA-TRAK assay ($p < 0.0007$). Similarly, the sensitivity of 5-ALA fluorescent cytology was significantly higher in high-grade lesions when compared to conventional cytology ($p < 0.0005$) and the BTA-TRAK assay ($p < 0.039$) (►**Fig. 3** and ►**Table 4**).

Discussion

A precursor of hemoglobin and chlorophyll, 5-ALA is a naturally occurring amino acid necessary for heme synthesis. The enzymatic activity of the heme synthesis pathway is altered in cancerous cells. The heme precursor PpIX accumulates in cells upon 5-ALA administration.[17,18] 5-aminolevulinic acid is non-fluorescent; however, 5-ALA-induced PpIX radiates red fluorescence when illuminated with blue/violet light, which enables its use for specific tumor detection.[19,20] This scientific concept has been used in the diagnosis of flat lesions associated with papillary carcinomas and CIS that could be overlooked during WLC. In 1994, Kriegmair et al.[21] reported that photodynamic diagnosis (PDD) following intravesical instillation of 5-ALA was possible and superior to traditional WLC in detecting flat lesions and CIS. In 15 patients, 26 neoplastic lesions were diagnosed

Table 2 Results of various tests and comparison to histopathology

Parameters	Conventional cytology	5-ALA fluorescent cytology	NMP-22 assay	BTA-TRAK assay
Positive for malignancy: imaging-confirmed bladder cancer (n = 150): n (%)	93 (62%)	136 (90.66%)	114 (76%)	110 (73.3%)
False negative: n (%)	57 (38%)	14 (9.3%)	36 (24%)	40 (26.67%)
False positive: n (%)	2 (1.34%)	6 (4%)	10 (6.67%)	8 (5.34%)
Sensitivity: %	62	90.6	76	73.3
Specificity: %	98.67	96.0	93.33	94.67
p-value	0.0001	–	0.0006	0.0001
Diagnostic accuracy: %	80.33	93.33	84.67	84.00
Cohen Kappa	0.62	0.842	0.724	0.7

Abbreviations: 5-ALA, 5-aminolevulinic acid; BTA-TRAK, bladder tumor antigen; NMP-22, nuclear matrix protein 22.

Table 3 Specificity of various tests and biomarkers in relation to grade and tumor (T) stage

Test	Tumor grade		Pathological T stage					Total
	Low	High	Ta	Tis	T1	T2	> T2	
Number (n)	**92**	**58**	**44**	**19**	**42**	**32**	**13**	**150**
Conventional cytology: n (%)	52 (56.5)	41 (70.6)	25 (56.8)	10 (52.6)	25 (59.5)	23 (71.8)	10 (76.9)	93
5-ALA: n (%)	81 (88.02)	55 (94.8)	39 (88.6)	16 (84.2)	38 (90.4)	31 (96.8)	12 (92.3)	136
NMP-22 assay: n (%)	65 (67.3)	49 (84.4)	32 (72.7)	10 (52.6)	35 (83.3)	27 (84.3)	10 (76.9)	114
BTA-TRAK assay: n (%)	62 (67.3)	48 (82.7)	30 (68.1)	9 (47.3)	35 (83.3)	26 (81.2)	10 (76.9)	110

Abbreviations: 5-ALA, 5-aminolevulinic acid; BTA-TRAK, bladder tumor antigen; NMP-22, nuclear matrix protein 22.

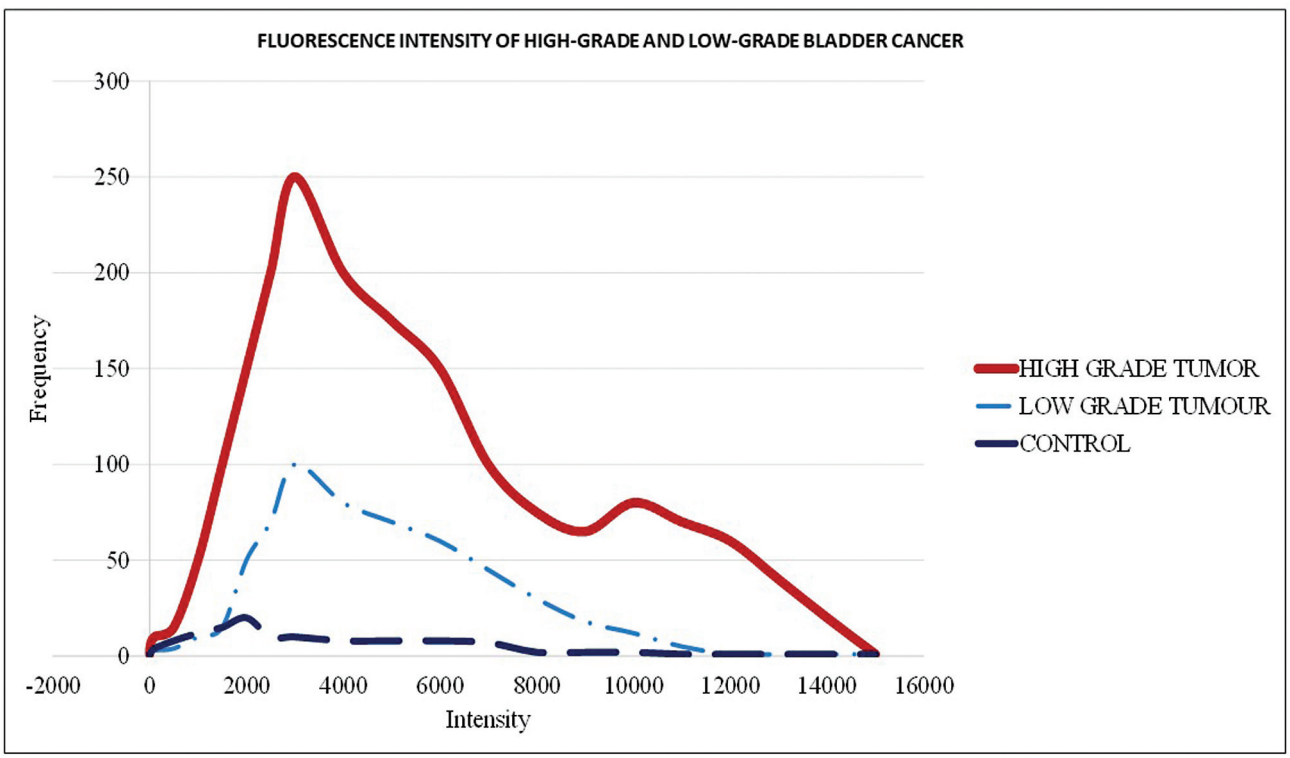

Fig. 3 Cytology induced by 5-ALA on high- and low-grade tumors.

Table 4 Grade of the tumor and sensitivity

Diagnostic test	Bladder cancer			
	Tumor grade			
	Low (n = 92)	*p*-value	High (n = 58)	*p*-value
5-ALA: n (%)	**81 (88.02)** *	Reference	**55 (94.8)** *	Reference
Conventional cytology: n (%)	52 (56.5)	0.0001*	41 (70.6)	0.0005*
NMP-22 assay: n (%)	65 (70.6)	0.0035*	49 (84.4)	0.067
BTA-TRAK assay: n (%)	62 (67.3)	0.0007*	48 (82.7)	0.039

Abbreviations: 5-ALA, 5-aminolevulinic acid; BTA-TRAK, bladder tumor antigen; NMP-22, nuclear matrix protein 22.

only by PpIX fluorescence, suggesting that 5-ALA-PDD could support the complete resection of bladder tumours.[21,22] In 2003, a multicentric study demonstrated that HAL hydrochloride was effective in detecting superficial bladder cancer, especially CIS.[23]

Based on this observation, several preliminary studies[13,15,24,25] were conducted on the use of 5-ALA/PpIX fluorescent urine cytology to enhance the detection of bladder cancer. Fluorescent urine cytology detects bladder cancer cells by observing PpIX fluorescence after ex-vivo incubation of the collected urine specimen with 5-ALA. The results of these studies[13,15,24] showed a significant difference compared to conventional urine cytology, especially in low-grade bladder cancers. In their preliminary study, Shadab et al.[13] evaluated the efficacy of 5-ALA-induced fluorescent urine cytology and reported that it was a highly-sensitive test to diagnose bladder cancer and that it showed a significant difference, especially in low-grade bladder cancer, when compared to conventional cytology. This new non-invasive detection technique that uses the 5-ALA/PpIX fluorescent urine cytology system that can be used in routine clinical practice is expected to gain momentum in the future.

The present study clearly shows that 5-ALA-induced fluorescent urine cytology is a very sensitive test in the diagnosis of bladder cancer. Irrespective of the grade of the lesion, the sensitivity remained high. Compared to the other non-invasive means of diagnosing bladder cancer, 5-ALA-induced fluorescent urine cytology was superior to conventional cytology and the BTA-TRAK and NMP-22 assay, which is in line with reports by other authors (►**Table 5**).[13,15,24] The current is probably the only study in which the results of 5-ALA-induced fluorescent urine cytology have been compared with those of conventional cytology and the BTA-TRAK and NMP-22 assays.

These tests with voided urine samples could be of use in many instances in the clinical practice. A cytological diagnosis of malignancy or cancer can be established in cases in which the patient is not fit enough to undergo conventional cystoscopy and biopsy. This test could be more helpful in patients with non-muscle invasive bladder cancer (NMIBC) who are on follow-up and need to undergo a check cystoscopy once every three months. Check cystoscopy could be avoided in patients with negative results on 5-ALA-induced fluorescent urine cytology. The results of the present study needs to be further validated by multicentric studies so as to consider it as a standard of care in the clinical setting.

Conclusion

Fluorescent urine cytology induced by 5-ALA is a highly-sensitive test in the diagnosis of cancer of the bladder when compared to conventional cytology and the BTA-TRAK and NMP-22 assays. The specificity of this test is similar to that of conventional cytology, and its advantages are that it is simple to perform, reliable and reproducible. Moreover, the test requires a voided urine sample, making it non-invasive, without complications and financially viable.

Authors' Contribution

RBN: conception and design, final approval of the manuscript, manuscript writing, and provision of study materials or patients; SR: collection and assembly of data, data analysis and interpretation, and final approval of the manuscript; SCG: conception and design, data analysis and interpretation, final approval of the manuscript,

Table 5 Comparison of various studies using 5-ALA-induced fluorescent urine cytology

Authors	Year	Patients: n	Sensitivity (%)	Specificity (%)
Miyake et al.[15]	2014	58	86.2	70.6
Yamamichi et al.[24]	2019	104	88.4	100
Shadab et al.[13]	2021	25	100	98.67
Present study*	2023	150	90.6	96.32

Abbreviation: 5-ALA, 5-aminolevulinic acid.

manuscript writing, and provision of study materials or patients; PL: data analysis and interpretation and final approval of the manuscript; SC: collection and assembly of data, data analysis and interpretation, and final approval of the manuscript; SC: collection and assembly of data, data analysis and interpretation, final approval of the manuscript, and manuscript writing.

Clinical Trials

None.

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Conflict of Interests

The authors have no conflict of interests to declare.

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