Original Article

The rapid test to determine the urine urease activity in children with urolithiasis

Assel Sagymbayeva[1], Natalya Merkusheva[2], Minira Bulegenova[3], Abay Kussainov[4], Bakitzhan Abekenov[5], Anar Musabalina[6]

ABSTRACT

Objective

To study the clinical efficiency of the newly proposed Rapid Urine Urease Activity (UA) test in detecting urease–producing bacteria responsible for the formation of kidney calculi in children with urolithiasis.

Materials and Methods

The prospective observational study involved 80 children: 40 with urolithiasis and 40 healthy children. The urolithiasis patients were divided into three subgroups based on their urease activity. All patients underwent the Rapid Urine UA test, standard urinalysis, standard bacteriological urine test, metabolic disorder analysis in urine, and chemical analysis of removed stones.

Results

Urine UA levels in healthy children were 0 [0-10] mmol/l, while in urolithiasis patients, they were 57 [50-200] mmol/l. Maximum UA levels were observed when detecting pathogens such as *Klebsiella pneumoniae* (340 mmol/l), *Proteus mirabilis* (300 mmol/l), and *Pseudomonas aeruginosa* (256 mmol/l). Medium UA levels (51-100 mmol/l) were recorded in patients with WBC of 23 [20-182] per mL, uric acid crystals, and acidic pH (4). Low UA levels (21–50 mmol/l) were observed in patients with no crystals in urine, a slight increase in WBC 22 [16-24] per mL. Patients in this subgroup, compared to others, exhibited disorders like hypercalciuria (25%) and hyperoxaluria (22.5%), with calcium oxalate stones in most cases. *Proteus mirabilis* had UA levels ranging from 58 mmol/l up to 300 mmol/l; *Pseudomonas aeruginosa* from 100 mmol/l up to 256 mmol/l; *E. coli* from 50 mmol/l up to 94 mmol/l; *Klebsiella pneumoniae* from 309 mmol/l up to 340 mmol/l.

Conclusion

The rapid urine UA test is a clinically efficient method for identifying active urease-producing bacteria in urine, contributing to the formation of infected stones in children.

Keywords

urolithiasis; urinalysis; urease activity; crystals; uropathogens; kidney stone formation.

INTRODUCTION

In many cases, the urine of patients with urolithiasis is infected with various bacteria[1-6], with the most common being *E. coli, Proteus mirabilis, Staphylococcus aureus*, and *Pseudomonas aeruginosa*[7]. Several bacteria produce the enzyme urease, and its activity varies depending on the type of bacteria. Urease plays a pivotal role in the formation of infected stones, predominantly struvite and apatite. This stands as one of the primary mechanisms for crystal formation in infected urine, leading to the subsequent formation of calculi in the kidneys[2,17,18,26].

Despite the prevalence of bacterial infection in urolithiasis patients, the rapid Urine UA

1. Assel Sagymbayeva, department of Pediatric Urology, Scientific Center of Pediatrics and Pediatric Surgery, Almaty city, Republic of Kazakhstan.
2. Natalya Merkusheva, Independent researcher, Canada.
3. Minira Bulegenova, department of Pediatric Urology, Scientific Center of Pediatrics and Pediatric Surgery, Almaty city, Republic of Kazakhstan.
4. Abay Kussainov, department of Pediatric Urology, Scientific Center of Pediatrics and Pediatric Surgery, Almaty city, Republic of Kazakhstan.
5. Bakitzhan Abekenov, department of Pediatric Urology, Scientific Center of Pediatrics and Pediatric Surgery, Almaty city, Republic of Kazakhstan.
6. Anar Musabalina, department of Pediatric Urology, Scientific Center of Pediatrics and Pediatric Surgery, Almaty city, Republic of Kazakhstan.

Correspondence

Dr. Assel Sagymbayeva, pediatric surgeon, Pediatric Urology Department, Scientific Center of Pediatrics and Pediatric Surgery, Almaty city, Republic of Kazakhstan, E-mail address: sagymbaeva.assel@gmail.com

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test has not been widely utilized in standard pediatric practice due to a lack of information about this method. Developed in 1994 at the National Center of Urology in Almaty, Kazakhstan, this method was initially applied exclusively in adult patients with urolithiasis[8,9,10].

Traditional urological practices lack standardized tests for determining the presence and activity of urease directly in urine via spectrophotometry. Most existing tests are designed to detect urease activity in isolated bacteria, presenting limitations and not being widely adopted in clinical urological practice. Many of these tests are primarily suited for research purposes due to their expensive cost and time-consuming nature[11,12].

The proposed spectrophotometric rapid Urine UA test offers several advantages, including its low cost, accessibility for any laboratory, and quick results within one hour. This stands in stark contrast to the standard bacteriological test, which requires several days up to one week for results. In the absence of a standard procedure for directly detecting urease activity in urine, we compared the rapid spectrophotometric urine UA test with the standard bacteriological test and urinalysis.

Our goal was to assess the clinical efficiency of the rapid Urine UA test in children with urolithiasis and its advantage in detecting recurrent calculi formation in the kidneys.

MATERIALS AND METHODS

The prospective observational study was conducted in the Scientific Centre of Pediatrics and Pediatric Surgery, Almaty, Kazakhstan from September 2021 to July 2023. Following approval from the ethical committee (protocol 1102/2021) and securing written informed consent from the patients, the study enrolled 40 children diagnosed with obstructing stone of the upper urinary tract (the main group) and 40 children who were considered conditionally healthy (the control group).

All patients diagnosed with a confirmed blockage caused by a stone in the upper urinary tract through clinical examination and tests like abdominal X-ray and ultrasound of the abdomen and urinary tract (detecting stones ≥7 mm) were part of the main group. Some patients with urolithiasis were not considered for the study, including those not needing surgery, those who declined participation or didn't attend follow-up appointments, and those from whom urine was collected through a urethral catheter or nephro-/pyelostomy.

The control group consisted of children in good health undergoing routine checkups to ensure they had no urinary tract or kidney problems. A comparison was made between the two groups regarding gender, age, and body mass index (BMI).

All children in both groups collected their morning urine in full volume, excluding the first portion. The urine was collected in a dry, clean container for both the rapid urine UA test and the standard urinalysis. The rapid urea UA test was conducted within 1 hour of urine collection. Urine for the bacteriological test was collected separately using the standard procedure. For the analysis of calcium, oxalate, uric acid, and citrate, the urine of patients was collected over a 24-hour period

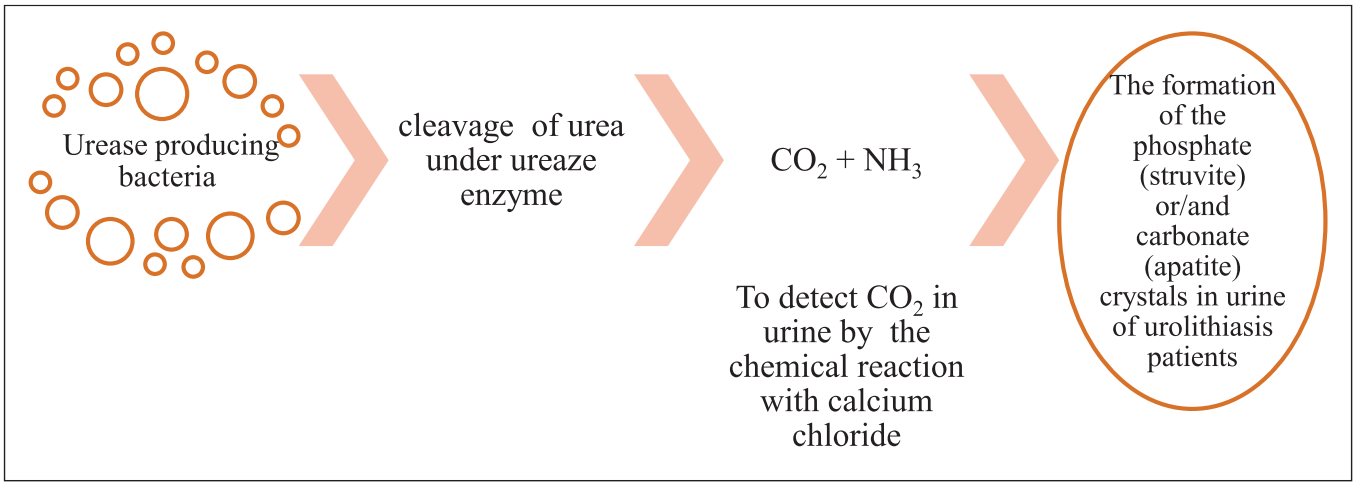

Figure 1: The Rapid Test to Determine the Urine Urease Activity.

and analyzed using reagents from Biosystem's (Spain) method on Abbot Architect c4000 (USA).

Indicators such as pH, WBC, RBC, bacteria, and crystals were obtained from the standard urinalysis using the Uriscan strip-test (Germany) for pH and microscopy for the other indicators. Bacteriological examination of urine was performed by calculating the number of colonies forming on 5% blood agar or Cled agar divided by sectors[3,27].

Chemical analyses of removed stones were conducted in a specialized laboratory. Patients were solely responsible for delivering the stones to the laboratory and sharing the obtained results with us. Unfortunately, not all results were available to us due to various reasons.

The rapid UA test is based on a chemical reaction between the product of urine disintegration by the urease enzyme and calcium chloride. The principle of the method is illustrated in Figure 1.

The presence of urease-producing bacteria in the urine leads to the depletion of urea, resulting in the formation of carbon dioxide and ammonia. This process, in turn, causes an increase in pH, leading to the crystallization of struvite and/or apatite. The crystallization process is dependent on the presence of calcium and phosphate cations in the urine. The procedure is conducted at a temperature of 37.0°C in a laboratory thermostat. To prevent bacterial contamination of the urine, a sterile 10% $CaCl_2$ solution is used.

Spectrophotometric measurement of the released $CaCO_3$ in urine is carried out at a wavelength of 691 nm. Extinction measurements are taken twice: before incubation and after the addition of $CaCl_2$. The sample is incubated for 1 hour[8,9,10].

This test reveals the presence of any urease-producing bacteria in urine within about an hour, whereas the routine bacteriological test may take from a few days up to one week. The laboratory can easily conduct this test using regular equipment such as the spectrophotometer and the lab thermostat. While the test is not specific and does not provide the exact names of bacteria in urine, it detects the activity of the urease enzyme. It proves to be a valuable tool for predicting crystal formation in urine and, subsequently, the formation of infected stones in the kidney.

The rapid UA test is the only clinical test used for spectrophotometrically detecting the activity of urease directly in urine. Therefore, it cannot be directly compared with similar tests but can be compared with standard urinalysis parameters and the bacteriological test.

The main group (40 children with urolithiasis) was divided into three subgroups depending on the level of urine urease activity: the first with 22 patients exhibiting low levels of UA (11-50 mmol/l), the second with 7 patients showing a medium increase in UA (51-100 mmol/l), and the third with 11 patients having very high levels of UA (101-400 mmol/l).

Statistical analyses

Statistical analysis required the use of software StatTech v. 2.8.8 (Developer - StatTech LLC, Kazan). The normality of quantitative variables was examined using the Shapiro-Wilk test. Quantitative variables conforming to a normal distribution were described using the mean (M) and standard deviation (SD), along with a 95% confidence interval (95% CI). For those not adhering to a normal distribution, descriptions included the median (Me) and interquartile range (IQR).

Categorical data were represented with absolute values and percentages. Group comparisons for non-normally distributed quantitative measures were performed using the Kruskal-Wallis test. Percentage comparisons in multifield matrix analyses were conducted using Pearson's chi-square test. The correlation between two quantitative variables, especially in cases of non-normal distributions, was assessed using the Spearman rank coefficient. Additionally, a linear regression method was employed to develop a predictive model describing the relationship between a quantitative variable and its influencing factors.

RESULTS

The main and control groups of patients did not significantly differ in terms of age, gender, and BMI. In the main group, there were 24 (60%) boys and 16 (40%) girls, while the control group consisted of 20 (50%) boys and 20 (50%) girls. Among patients with urolithiasis, the age ranged from 3 months to 5 years.

In patients with urolithiasis, the average size of the obstructing stone was 20 ± 3 mm, and the Hounsfield stone density was 2050 [1800 - 2625]. The left kidney was affected in 23 (57.5%) patients, and the right kidney in the remaining 17 (43.5%) patients.

Urine urease activity (UA) was significantly increased

Table 1: The standard urine indicators and UA in healthy children and urolithiasis patients

The standard urinalysis	Children with urolithiasis N=40			Healthy children	P Value*
	1st subgroup UA 11-50 mmol/l N=22	2nd subgroup UA 51 - 100 mmol/l N=7	3d subgroup UA 101-400 mmol/l N=11	UA 0 - 10 mmol/l N=40	
pH, Median [Q_1 – Q_3]	6.00 [6.00-6.00]	4.00 [4.00-6.00]	6.00 [6.00-7.00]	6.00 [5.50-6.00]	0,004*
White blood cells / WBC per mL, Median [Q_1 – Q_3]	22 [16-24]	23 [20-182]	187 [32-292]	2 [1-2]	0,001*
Red blood cells /RBC per mL, Median [Q_1 – Q_3]	5 [2-11]	3 [2-20]	17 [3-34]	1 [0-1]	0,191
Bacteria	0	10^5	$>10^5$	0	0,001*
Presence of crystals in the urinalysis (number of cases)	0	7	11	0	0,001*

Notes: * – differences are statistically significant ($p < 0.05$). P-value was calculated by chi-square test or Kruskal-Wallis test.

(< 0.001) in the group of urolithiasis patients (N=40) compared to the group of healthy children (N=40), with values of 57 [50-200] mmol/l and 0 [0-10] mmol/l, respectively.

The obtained results of urinalysis are presented in Table 1.

Indicators of urease activity depending on the type of isolated microorganisms are presented in Figure 2.

According to the data obtained from the comparison of urease activity, statistically significant differences were revealed depending on the pathogen spectrum of urinary tract infections ($p < 0.001$) using the Kruskal-Wallis test.

In the urine of healthy children (N=40), uropathogens were absent, and UA ranged from 0 up to 10 mmol/l. Meanwhile, in the urine of urolithiasis patients (N=40), various uropathogens were present, and UA ranged from 48 mmol/l up to 340 mmol/l (Figure 2).

Uropathogens produce the urease enzyme with varying activity (Table 2). In our study, we found that the highest urease activity (UA) was observed in the presence of *Klebsiella pneumoniae* (316 mmol/l). Moderate UA levels (101 mmol/l) were associated with *Pseudomonas aeruginosa* and a combination of two or more bacteria (84 mmol/l). The lowest UA levels were found in urine with *Proteus mirabilis* (62 mmol/l), E. Coli (54 mmol/l), *Enterococcus faecalis*, and *Staphylococcus haemolyticus*. These findings are consistent with other sources[4,5,6].

It is important to note that the same bacterium might produce urease with lower or higher activity. For example, *Proteus mirabilis* exhibited UA levels ranging from 58 mmol/l up to 300 mmol/l, *Pseudomonas aeruginosa* from 100 mmol/l up to 256 mmol/l, and E. coli from 50 mmol/l up to 94 mmol/l. These results align with other information sources, where scientists discovered in urolithiasis patients that *Proteus mirabilis* and *Providencia stuartii* showed synergistic induction of urease activity[13].

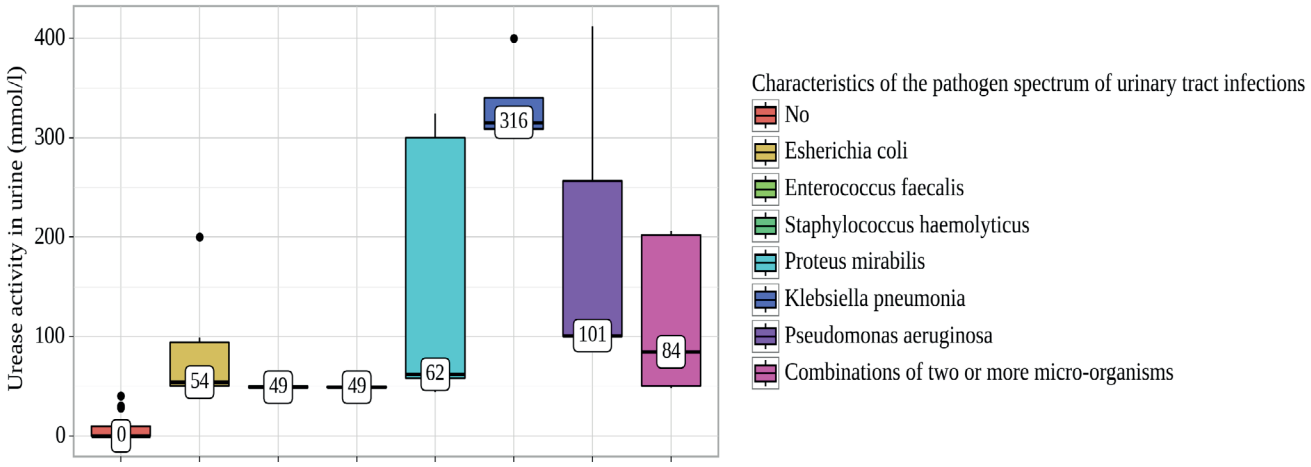

Figure 2: UA Regarding to the Urinary Tract Infections.

Table 2: Distribution of urine pathogens in groups of urolithiasis patients

Urinary tract infections	Urolithiasis patients N=40		
	1st subgroup UA 21-50 mmol/l N=22	2nd subgroup UA 51- 100 mmol/l N=7	3d subgroup UA 101- 400 mmol/l N=11
E. Coli	7 (17.5%)	2 (5%)	0
Enterococcus faecalis	8 (20%)	0	0
Staphylococcus haemolyticus	1 (2.5%)	1 (2.5%)	0
Proteus mirabilis	3 (7.5%)	1 (2.5%)	3 (7.5%)
Klebsiella pneumonia	0	0	4 (10%)
Pseudomonas aeruginosa	0	2 (5%)	1 (2.5%)
Combination of two or more microorganisms	3 (7.5%)	1 (2.5%)	3 (7.5%)

Table 3: The chemical composition of urine crystals in urolithiasis patients obtained by standard urinalysis

Chemical composition	2nd subgroup UA 51 – 100 mmol/l N = 7	3d subgroup UA 101 – 400 mmol/l N = 11
Uric acid	7 (39%)	-
Triple phosphates or amorphous phosphates	-	5 (28%)
Calcium oxalate + uric acid/or ammonium biurate	-	6 (33%)

Patients in the 1st subgroup did not exhibit any crystals in their urine, as confirmed by the standard urinalysis. Consequently, they were not included in Table 3. The results of standard 24-hour urine tests, which reveal metabolic disorders, have been included in Table 4.

Table 4: The compared analysis of metabolic disorders and UA in groups of urolithiasis patients

Metabolic disorders	Number of cases		
	1st subgroup UA 21-50 mmol/l N=22	2nd subgroup UA 51-100 mmol/l N=7	3d subgroup UA 101-400 mmol/l N=11
Hypercalciuria	10 (25%)	-	-
Hyperoxaluria	9 (22.5%)	-	-
Hypocitraturia	1 (2.5%)	-	6 (15%)
Hyperuricosuria	-	5 (12.5%)	-
Hypercalciuria+ Hyperoxaluria+ Hypocitraturia	2 (5%)	2 (5%)	5 (12.5%)

Most urolithiasis patients exhibited hypercalciuria and hyperoxaluria, with hypocitraturia being associated with high urine UA; all 11 patients in the 3rd subgroup had it.

The chemical analysis of primary stones was conducted only for 23 urolithiasis patients due to the mentioned circumstances.

In the 1st subgroup, with the lowest UA in urine, all 11 stones consisted of calcium oxalate, whereas in the 3rd subgroup, with the highest UA, stones consisted of pure calcium phosphate or a combination of calcium phosphate and calcium oxalate (Table 5).

Table 5: The chemical composition of primary stones in urolithiasis patients

Chemical composition of primary stones	Number of cases		
	1st subgroup UA 11 -50 mmol/l N=11	2nd subgroup UA 51-100 mmol/l N=3	3d subgroup UA 101-400 mmol/l N=9
Calcium oxalate dihydrate	11 (47.8%)	-	-
Calcium phosphate	-	-	5 (21.8%)
Uric acid	-	2 (8.7%)	-
Calcium phosphate mixed with Calcium oxalate	-	1 (4.3%)	4 (17.4%)

DISCUSSION

Urolithiasis is a multifactorial disease with a high recurrence rate[14-17]. The absence of preventive measures often results in recurrence within 5 years in nearly 50% of urolithiasis patients. In more than 60% of cases, recurrences occur within three years after the removal of the primary stone[19,20,21]. Typically, the treatment of urolithiasis is confined to calculus removal, with insufficient attention given to monitoring recurrent kidney stone formation. One reason for this, in our opinion, is the lack of adequate laboratory tests indicating crystal formation in urine and, consequently,

kidney stone formation. Currently, there are only a few proposed tests[17,22], and research in this area is ongoing.

The urease activity of bacteria raises the pH of urine, allowing the precipitation of normally soluble polyvalent ions into struvite and carbonate apatite. These compounds aggregate around bacteria, forming urinary stones. Within such stones, microorganisms are protected from antibiotics and the host's immune system[11,26]. It has been observed that *Proteus mirabilis* infection caused ureolytic activity in the kidneys, leading to the formation of necrotic cells during acute inflammation. One week later, pyelonephritis was in progress, and struvite stones were detected. After two weeks, kidneys were ulcerated, and fibrosis was visible[11,26]. Moreover, ammonia released by urease causes damage to the glycosaminoglycan layer on the urothelial surface, impairing its protective function. The role of ureolytic activity in urinary stone formation has also been demonstrated for *U. urealyticum, S. saprophiticus, S. aureus*, some *Klebsiella spp.,* P*seudomonas spp.*, as well as *Corynebacterium sp.*, *P. penneri, P. stuartii, M. morganii*[11,26]. Bacterial ureases are considered to be major antigens in some human diseases, and persistent diseases caused by ureolytic bacteria may stimulate the generation of antibodies[11,26].

The Rapid UA test is a novel tool in the pediatric field for spectrophotometrically detecting the presence of urease-producing bacteria directly in urine and predicting the formation of infected stones in the kidneys of urolithiasis patients. All 40 urolithiasis patients were examined one month after surgical treatment to monitor the recurrence of calculi formation.

In our research, we observed that in the presence of urea-splitting bacteria, UA was higher than 10 mmol/l (Table 1; Fig. 2). Due to the moderate number of patients in each group, we decided to expand the reference range for UA and chose 21 mmol/l as the starting point for UA, indicating the presence of urea-splitting bacteria in urine, despite the lowest UA in our case being 48 mmol/l (Fig. 2). We divided all patients into three subgroups, with the 1st having UA from 21 mmol/l to 50 mmol/l, the 2nd from 51 mmol/l to 100 mmol/l, and the 3rd from 101 mmol/l to 400 mmol/l. This approach allowed us to explore the relationship between UA and routine lab indicators.

According to standard urinalysis, 22 patients in the 1st subgroup had normal pH, no crystals in urine, and no visual bacteria in urine. The absence of crystals indicated a low risk of recurrent calculi formation. UA was higher compared to healthy children (21-50 mmol/l and 0-10 mmol/l, respectively), with a WBC count of 22 [16-24] per mL. The bacteriological test detected the following urine pathogens: 7 children had *E. coli*, 8 had *Enterococcus faecalis*, 3 had *Proteus mirabilis*, 1 had *Staphylococcus haemolyticus*, and 3 patients had a combination of two or more bacteria in urine. 11 children from this subgroup had primary calcium oxalate calculi. Chemical analysis of calculi in the remaining 11 patients had not been performed. In this subgroup, 25% of patients had hypercalciuria, 22.5% had hyperoxaluria, and 5% had both metabolic disorders along with hypocitraturia. One person from this subgroup (5%) had hypocitraturia. Thus, 22 patients from the 1st subgroup had kidney stones developed due to metabolic disorders as the main reason, requiring correction of calcium and oxalate excretion through dietary adjustments, water intake, and other standard recommendations.

Despite the absence of crystals and visual bacteria in the standard urinalysis, we detected urease with low activity in the urine of these patients, as confirmed by the bacteriological test revealing the presence of *E. coli* and *Enterococcus faecalis* in most cases. This condition posed a risk for recurrent calculi formation, and these children, in addition to metabolic correction, needed mild urine sanitation, often with herbal remedies. Recent studies have revealed the presence of E. coli in the majority of kidney stones[23,24,26], challenging the old notion that only *Proteus mirabilis* was responsible for the formation of most kidney stones. In our research, we observed similarities in the 1st subgroup, where urine *E. coli* was present in most cases and associated with the formation of calcium oxalate stones.

Thus, the low UA in patients of the 1st subgroup correlated with normal urinalysis indicators and uropathogens that didn't produce high-activity urease enzyme.

The higher UA in the 2nd subgroup, accompanied by an increased WBC count of 23 [20-182] per mL, bacteriuria, and the presence of pathogens in urine such as *E. coli* with UA of 94 mmol/l, Pseudomonas aeruginosa with UA of 100 mmol/l, and Proteus mirabilis with UA up to 62 mmol/l.

Patients in the 2nd subgroup had crystals of uric acid, a urine pH of 4, and the composition of the primary stones in 2 patients included uric acid as well. The

examination of the stone chemical composition for the remaining 5 patients was not performed, but other lab tests were positive for it (Tables 3, 4, 5).

Thus, revealed correlation between UA urinalysis and the bacteriological test pointed that these patients needed beside the metabolic treatment some urine sanitation according to the lab tests and clinical symptoms.

In urine of the 3d subgroup of patients (11 children or 27.5% from the total number of patients) UA was the highest- from 101mmol/l up to 400 mmol/l. They also had the highest WBC 187 [32 – 292], bacteriuria (10>5), and urine pathogens with the highest UA: Klebsiella pneumonia -340 mmol/l, Proteus mirabilis-300 mmol/l, Pseudomonas aeruginosa -256 mmol/l. The urinalysis revealed the presence of inflammation and high risk for the recurrent kidney stone formation: pH up to 7, RBC 17 [3-34] per mL, the crystals of triple phosphate and calcium oxalate in association with uric acid (or in alkaline urine - ammonium biurate). The urolithiasis patients of this subgroup needed the antibacterial treatment with the antibiotics according to the results of the bacteriological tests.

Besides, some patients had hypocitraturia associated with the high UA. Our data corresponded the results by De Cogain et.al[25] which stated that some bacteria could produce the enzymes urease and citrate lyase simultaneously (Table 6).

Table 6: The enzyme activity based on typical species characteristics

Uropathogens	Urease production	Citrate hydrolysis
Pseudomonas aeruginosa	+	+
Klebsiella pneumonia	+	+
Proteus mirabilis	+	+/-

Thereby, 8 patients from 3d subgroup had all three uropathogens listed in Table 6, 3 patients from this subgroup had the combination of 2 or more bacteria in urine. In our opinion, the hypocitraturia in this patient was the result of citrate hydrolysis by *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, Proteus mirabilis, and probably combination of 2 and more bacteria. We thoroughly checked patient's 1st and 2nd subgroups and discovered that in the 2nd subgroup 2 patients had hypocitraturia and *Pseudomonas aeruginosa* in urine; in the 1st subgroup 3 patients had hypocitraturia *Proteus mirabilis*.

Thus, these data need further research to confirm that hypocitraturia in urine of urolithiasis patients is depended on the production of citrate lyase by *Pseudomonas aeruginosa, Klebsiella pneumonia, Proteus mirabilis.*

Thus, the deviations in UA rapid test corresponded alterations of urinalysis and the bacteriological test. Out of 11 patients of 3rd subgroup 9 children had the primary stones consisted of phosphates or mixed phosphates with calcium oxalates that was typical for the infected stones. These patients had hypercalciuria, hyperoxaluria together with hypocitraturia at the same time. Our research brought new facts about the activity of enzyme urease in urine of urolithiasis patients, thus the most common bacterium Proteus mirabilis had whole range of UA starting from 62 mmol/l up to 300 mmol/l. If low UA was associated with the formation of uric acid crystals, then the high UA was typical for the infected stones.

Thus, the Rapid Urine UA Test is very helpful lab method to detect the presence of urease-producing bacteria in urine of children with urolithiasis, and it is very helpful tool in regular clinical practice to prevent a recurrence of urolithiasis. It has advantages due to its accessibility for all types of laboratories because it doesn't require any additional equipment, expensive reagents and very rapid compared to the standard bacteriological test. This test has some limitations because it doesn't provide the exact name of urine pathogen and gives only the activity of enzyme urease that might come to urine from different bacteria.

This test is convenient to screen urine for the presence of any urea-splitting bacteria, but it doesn't eliminate the standard bacteriological test, which provides the information about the exact pathogen and its quantity.

Limitations of the study

Our study had some limitations; it did not include a very large group of children with urolithiasis. In the first phase of our work, we concentrated on the studying the advantages of UA test, if it was informative, useful, correlated with other standard parameters.

Obtained results presented the first phase of our work, we emphasized to confirm the advantages of the Rapid UA Test. We included patients with a high risk of recurrent

stone formation; all 40 patients had the calculi, located in the upper third of the urinary tract, that caused renal colic, inflammation with all its consequences, and who mostly needed the urgent treatment.

Due to very high risk of urolithiasis recurrence, we are planning to continue the regular monitoring this group of patients to predict and prevent the process of kidney stone formation.

CONCLUSION

The Rapid Urine UA Test is a valuable tool to predict the risk of kidney stone formation revealing the active urease-producing bacteria in urine, that contribute to the formation of infected stones.

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Conflict of Interest

The authors declare no conflicts of interest.

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Data Availability

This is an original paper. All data are available for only research purposes from principal investigators.

Authors' Contributions

The authors confirm contribution to the paper as follows: study conception and design: AS, NM; data collection: AS, MB; analysis interpretation of results: AS, NM, AM, AK, draft manuscript preparation: AS, NM, BA. All authors reviewed the results and approved the final version of the manuscript.

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