Review

Joris Delanghe*, Marijn Speeckaert, Sigurd Delanghe and Matthijs Oyaert

Pitfalls in the diagnosis of hematuria

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Abstract: Detection of hemoglobin (Hb) and red blood cells in urine (hematuria) is characterized by a large number of pitfalls. Clinicians and laboratory specialists must be aware of these pitfalls since they often lead to medical overconsumption or incorrect diagnosis. Pre-analytical issues (use of vacuum tubes or urine tubes containing preservatives) can affect test results. In routine clinical laboratories, hematuria can be assayed using either chemical (test strips) or particle-counting techniques. In cases of doubtful results, Munchausen syndrome or adulteration of the urine specimen should be excluded. Pigmenturia (caused by the presence of dyes, urinary metabolites such as porphyrins and hemoglutaminic acid, and certain drugs in the urine) can be easily confused with hematuria. The peroxidase activity (test strip) can be positively affected by the presence of non-Hb peroxidases (e.g. myoglobin, semen peroxidases, bacterial, and vegetable peroxidases). Urinary pH, haptoglobin concentration, and urine osmolality may affect specific peroxidase activity. The implementation of expert systems may be helpful in detecting preanalytical and analytical errors in the assessment of hematuria. Correcting for dilution using osmolality, density, or conductivity may be useful for heavily concentrated or diluted urine samples.

Keywords: bladder cancer; expert systems; hematuria; peroxidase; pigmenturia.

Introduction

Hematuria is an important clinical symptom and key sign in diagnosing urological and nephrological diseases [1–3]. Although diagnosing hematuria might appear simple at first sight, clinicians and laboratorians may be easily misled when assessing hematuria due to the occurrence of false positive and negative results, even when modern urinalysis techniques for detecting hemoglobin (Hb) and red blood cells (RBCs) have been applied. A positive peroxidase reaction on a urinary test strip often leads to a false-positive diagnosis of hematuria or pseudo-hematuria. Consequently, investigating pseudo-hematuria may lead to unnecessary healthcare costs [4, 5].

Commonly used test strips incorporate a benzidine compound reduced with buffered organic peroxide (3,3',5,5'-tetramethylbenzidine, and diisopropylbenzene dihydrogenperoxide). A color reaction occurs when urine reacts with a test strip that includes an oxidizing substrate. For example, the pseudoperoxidase activity of Hb oxidizes benzidine compounds, thereby turning the reaction pad blue [6]. In contrast, urine particle analysis allows for the detection of RBCs in urinary specimens. In recent years, classical urine sediment has been largely replaced by automated urinalysis based on pattern recognition by automated microscopy or urinary (fluorescence) flow cytometry (UFFC) [7].

The introduction of sensitive analytical equipment [7] has allowed the implementation of lower diagnostic threshold values, which require stricter pre-analytical care of urine specimens [8]. However, chemical test strips and RBC particle analyses may provide discordant results. In this review, the most critical preanalytical, analytical, and post-analytical pitfalls in the assessment of hematuria are discussed.

Preanalytical issues

Sampling

The introduction of analytical methods characterized by a low coefficient of variation highlights the importance of the pre-analytical phase [8]. The pre-analytical phase of urinalysis can be divided into different subphases, including patient preparation with instructions, urine collection, pre-mixing of the urine in the collection container, subsequent transfer of the urine to the transfer tube, transportation of the transfer tube to the clinical
laboratory, and preservation of the device in the clinical laboratory. The ECLM European urinalysis guideline describes further details of urinary specimen collection to minimize errors [9].

Urine specimens can be transported at 20 °C on the day of collection if preserved properly. Longer delays require careful planning with the current preservatives. The use of preservatives for stabilizing urine specimens during transport may also affect results of urine particle analysis [10]. As UFFC is sensitive to non-dissolved preservative remnants, a background signal in the RBC channel may falsely increase the RBC counts [11]. Refrigeration increases various precipitates that may obscure RBC counts [10]. In addition, the use of vacuum urine transfer tubes may result in a significant increase in RBC count [11]. The vacuum applied in the urine collection tube and the small inner diameter of the needle in the collection container may affect the integrity of brittle RBC casts, resulting in cast disintegration and a falsely increased RBC count [11, 12].

Tampered urine specimens

Adulteration is a practice involving the manipulation of a urine specimen with chemical adulterants to produce a false-negative test result and is a major challenge in urine drug testing [13]. In case of adulteration, multiple doubtful results should be suspected. As urine can be easily tampered, cases of Munchausen syndrome presenting with red urine have been reported [13, 14]. Non-human Hb (e.g. meat juice) shows peroxidase activity comparable to that of human Hb, and factitious hematuria can be detected using additional testing. The morphology of non-human RBCs differs from that of human RBCs. When dealing with hemoglobinuria, protein electrophoresis of urine specimens may be helpful in establishing the presence of non-human proteins in urine specimens [13]. In an extreme case of Munchausen-by-proxy, a DNA Short Tandem Repeat test was chosen as a diagnostic tool for these patients [15].

Pigmenturia, a misleading sign

Hemoglobin is characterized by a typical reddish color with maximal absorbance at a wavelength of 415 nm. The high molar extinction of Hb allows sensitive detection of hematuria by simple visual inspection of the urine specimen. Gross hematuria is defined as the presence of a sufficient quantity of blood in the urine that is visible to the naked eye. Macroscopic hematuria roughly corresponds with the presence of more than 0.5 mL blood/L urine (±2500 RBC/µL or 2.5 10⁹ RBC/L) [16]. However, the presence of non-Hb reddish pigments in urine (pigmenturia) can be easily confused with hematuria [17]. Various causes of pigmenturia have been described.

Alkaptonuria is a genetic disease characterized by the inability of the homogentisate 1,2 dioxygenase enzyme to metabolize homogentisic acid [18]. Excess homogentisic acid is eliminated into the urine by the kidneys [19], which results in dark-colored urine. The urine test for homogentisic acid is the gold standard for diagnosing alkaptonuria [18]. The amount of homogentisic acid excreted daily in patients with alkaptonuria ranges between 1 and 8 g.

Betacyanins are a very common cause of pigmenturia. These red beetroot pigments may appear in urine following the consumption of beetroot (beeturia) [20]. The typical color can range from pink to deep red, and this phenomenon is prevalent in 10–14 % of the population following the consumption of beetroot. In addition, ingestion of red-colored cosmetic dyes (e.g. lip sticks) may mimic hematuria [21]. Furthermore, administration of a topical antiseptic containing azosulfamide (C₁₈H₁₄Na₂O₁₀S₃) may result in pigmenturia. Hydrophobic molecules are readily absorbed topically and excreted primarily in the urine [22]. In addition, the administration of certain drugs (e.g. phenazopyridine, rifampicin, vitamin B12, and fenytoin) may cause pigmenturia [23].

Hydroxocobalamin (the hydroxylated active form of vitamin B12 which is used for the treatment of cyanide poisoning) also causes reddish discoloration of the urine, mimicking hematuria [24]. This type of pigmenturia can persist up to 35 days after intravenous infusion [25]. Also, uric acid can result in a reddish coloration of diapers [26].

Finally, porphyria may be accompanied by pigmenturia [27]. Additionally, the presence of bile pigments in urine may cause pigmenturia [17]. Dark brown urine, due to excreted bilirubin, should be considered a differential diagnosis when hematuria or red-discolored urine is observed. Bilirubinuria is usually a result of conjugated hyperbilirubinemia from hepatocellular or post-hepatic causes [28]. In Table 1, a summary of the major causes of pigmenturia is presented.

Analytical issues

Pitfalls in measurements on chemical strips

The catalytic properties of Hb, a pseudoperoxidase, are used for the sensitive detection of hematuria. Modern urine strips take advantage of the peroxidase activity of Hb to detect hematuria [16]. If intact RBCs do not lyse, speckles may be produced on the test pad [16].
Modern test strip readers show excellent within-run and between-run imprecision and an analytical sensitivity of $13.6 \times 10^6$ RBCs/L, which is much lower than that of macroscopic examination [16, 29]. An optimal activity of Hb peroxidase was observed within a pH range of 5.0–6.5 and false negative peroxidase results were observed in the presence of ascorbic acid (vitamin C) [30]. This problem has been solved by several vendors by adding iodate to the Hb peroxidase test strip pad. The addition of iodate prevents peroxidase oxidation by vitamin C, thereby significantly reducing the risk of false-negative results [31]. In contrast, false-positive results for Hb peroxidase have been observed in the presence of oxidizing cleaning substances (e.g. hypochlorite) [16].

In addition to Hb, the heme-containing muscle protein myoglobin also expresses peroxidase activity. Given the low renal threshold for myoglobin, myoglobinuria may occur following muscular damage [32]. When the renal threshold for myoglobin is exceeded, myoglobinuria occurs, resulting in a peroxidase reaction. Consequently, myoglobinuria may result in pseudo-hematuria. Myoglobinuria can be distinguished from hemoglobinuria by differential microscopic examination [16, 29]. An optimal activity of Hb peroxidase was observed within a pH range of 5.0–6.5 and false negative peroxidase results were observed in the presence of ascorbic acid (vitamin C) [30]. This problem has been solved by several vendors by adding iodate to the Hb peroxidase test strip pad. The addition of iodate prevents peroxidase oxidation by vitamin C, thereby significantly reducing the risk of false-negative results [31]. In contrast, false-positive results for Hb peroxidase have been observed in the presence of oxidizing cleaning substances (e.g. hypochlorite) [16].

The contribution of non-heme peroxidase from bacterial sources may be important in the case of bacteriuria and may result in a false-positive Hb peroxidase reaction. Hydroperoxidase activity was detected in 80 % of isolates (in 27 of 27 g-negative bacilli cultures, in 6 of 6 staphylococci cultures, and in 3 of 12 streptococci cultures) on agar medium, and in 69 % of isolates in urine [36]. In addition to bacterial peroxidase, vegetable peroxidase sources have also been identified as potential interfering enzymes [17]. In addition, diluted povidone-iodine (betadine) solution, used to prepare a urethral meatus before the collection of urine cultures, may cause false-positive peroxidase test results [37].

In men, postcoital urine may be falsely positive for microhematuria. Since Hb and myoglobin are not found in normal human semen, it has been assumed that other seminal proteins that have heme groups or peroxidase activity catalyze the reaction that produces color on dipsticks. Cytochromes and/or antioxidant enzymes may play a role in chemical reactions leading to hematuria [38].

As peroxidase activity is characterized by a pH optimum and given the fact that urinary pH shows a marked variation (urinary pH may vary between 5 and 9), urinary pH values may affect peroxidase activity [6]. On the other hand, when proteinuria is concomitantly present, Hb can be bound to haptoglobin (Hp, particularly in subjects carrying a Hp 1–1 or Hp 2–1 phenotype which is characterized by a lower molecular mass) and enhance the specific peroxidase activity on urinary test strips [6, 39].

Non-standardized reading of urine test strips (typically carried out at point-of-care, especially with visual reading) is not recommended because of false positive results (overtime strip reactions, or urgency of testing of too warm urine specimens at body temperature rather than the specified 20 °C).

**Pitfalls in the RBC detection**

Manual microscopy is not a single method, but contains several versions of measurement procedures [40]. A standardized operating procedure has been recommended both as a reference microscopy, and for routine urine microscopies in international guidelines [9, 40].

Automated UFFC analyzers show non-optimal specificity for the detection of RBCs compared with manual microscopy. They can recognize, count, and classify particles by analyzing the forward scatter, side scatter, side fluorescence and depolarized side scattered light of stained particles. The principle is based on a 488-nm blue laser flow cytometry. Even with the introduction of depolarized side scattered light in the latest generation of UFFC analyzers, yeast-like cells can cause false-positive signals in the RBC channel. In addition, in some cases, calcium oxalate monohydrate crystals may cause positive interference [12, 40, 41]. These gaps in the detection of RBCs by UFFC can be overcome by implementing intelligent verification criteria (e.g. by concomitantly performing a urinary test strip analysis on the sample) [41–43].

<table>
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<tr>
<th>Table 1: Overview of the most common causes of pigmenturia.</th>
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<td><strong>Cause</strong></td>
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<td>Alkaptonuria</td>
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<td>Betacyanins</td>
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<td>Cosmetic dyes</td>
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<td>Urate</td>
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<td>Azosulfamide</td>
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<td>Drugs</td>
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<td>Hydroxocobalamin</td>
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<td>Porphyria</td>
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Urinary hypo-osmolality causes RBC lysis, which affects the morphological evaluation of RBCs. Automated microscopy urine particle analyzers fail to detect hemolyzed RBCs [44]. The implementation of phase-contrast optics in automated digital microscopy has improved ghost cell detection [45]. In contrast, flow cytometric techniques are less affected by RBC membrane damage, since the partially lysed RBC “ghost cells” can still be counted correctly by urine flow cytometry analysis [12].

**Quantification errors**

Manual microscopic urine sediment analysis is challenging because the number of particles counted is typically low. This implies that conventional manual counting techniques (counting chamber and urine sediment microscopy) are characterized by significant statistical errors owing to the Poisson distribution [46, 47]. In contrast, because of the higher number of counted particles during analysis, automated urinalysis is characterized by much lower coefficients of variation [40, 48].

Furthermore, counting uncentrifuged specimens suffers from low counts with high imprecision owing Poisson distribution, but they allow the best sensitivity of RBC detection when using phase-contrast optics [46]. Manual microscopy of concentrated urine sediment and resuspension suffers from losses, as not all RBC particles sediment during centrifugation because their density is equal to the urinary buoyant density, and become discarded with the supernatant [49].

Although dry chemistry technology for urinary test strips has made limited progress, advances in electronic detection have considerably improved the analytical sensitivity of test strip readers [6, 12]. When using this semi-quantitative interpretation, information on the concentration of the analyte in urine is lost. However, it has been demonstrated that the use of reflectance (or remission) data allows a more accurate expression of the test strip [6, 50].

**Post-analytical issues**

**Interpretation of test results**

The identification and correct quantification of RBCs is challenging for all automated urine particle analyzers for the following reasons: (1) the number of particles in urinary samples is often low; (2) RBCs may be present as isomorphic or dysmorphic RBCs, ghost cells, and/or acanthocytes in urine; (3) the shape and size of RBCs vary depending on the density of the urinary sample; and (4) automated urine particle analyzers may misclassify yeasts or crystals [40]. Comparison with manual microscopy, which is still the reference method, is difficult because the latter technique involves several methodological steps that may contribute to imprecision and inaccuracy [46, 51]. Therefore, combining urinary test strips and particle analysis, along with the implementation of manual microscopic review rules, improves the quality of automated UFFC particle analysis results, especially for RBCs [41].

Results from the direct chamber counting on native urine were comparable to those from UFFC when the total number of cells counted visually was statistically sufficient. The upper reference limit in health for RBCs determined by chamber counting on uncentrifuged, first morning urine is approximately $11 \times 10^6$ RBCs/L [52], and the conventional method of sediment microscopy may show 2–3 RBCs/high-power field (HPF) in healthy individuals [53].

**Use of expert systems**

Quantitative expression of the Hb peroxidase test strip results using reflectance data may be of great help in the development of urinary expert systems. Comparing the remission data of the peroxidase test pad of urinary test strips with RBC counts allows the detection of outliers caused by analytical errors of interfering substances. The implementation of expert systems, thereby improving the diagnostic performance of RBC detected by automated urine particle analyzers, allows the detection of analytical errors [12, 41–43]. Variation in sampling is a hurdle in assessing widely accepted reference ranges for hematuria [48]. In the assessment of dysmorphic RBCs, pitfalls include non-preserved specimens, lack and variability of observer skills in manual microscopy, and lack of complete detection by automated analyzers [54].

**Implementing measurands of urine concentration in test interpretation**

Finally, clinical urine specimens are characterized by a broad variation in density. The effects of urinary dilution affect RBC counts, as counts are usually expressed per volume of urine. This dilution effect can be compensated for by using measurands of urine concentration (osmolality, conductivity, and specific gravity) as corrective factors.
Conclusions

Despite the recent introduction of advanced methods in urinalysis, the diagnosis of hematuria is occasionally prone to pre-analytical and analytical errors. During the post-analytical phase, comparing urine test strip results (peroxidase reaction) with urine particle analysis (urine sediment) is mandatory in detecting analytical problems in hematuria detection. Expert systems may be useful in this context. For the correct interpretation of samples characterized by abnormal urine dilution, measurands of urine concentration (osmolality, conductivity, and specific gravity) may be of added value.

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