Centrifugation techniques and reagent strips in the assessment of microhaematuria

S. C. Freni1, G. J. Heederik2, and C. Hol1

SUMMARY During a survey for bladder cancer in a healthy male population a surprisingly high incidence of significant microhaematuria was found and no urinary sample was completely free of blood. A study of the sensitivity of the different techniques for assessing microhaematuria seemed necessary. A semiquantitative centrifugation technique, developed for cytological purposes, was found to be the most sensitive. Blood could be detected in concentrations of less than 50 RBC/ml urine. With conventional analysis, performed carefully, the minimum detectable concentration was about 500 RBC/ml. Reagent strips, based on the ortho-tholodin peroxidase reaction and developed specifically for urine analysis, gave positive reactions in minimum concentrations of 1 000 000 RBC/ml (original Hemastix), 50 000 RBC/ml (new Hemastix), and 20 000 RBC/ml (Sangur-Test). Positive scores with lower concentrations could be obtained with the Sangur-Test strip when it was read with a magnifying glass. However, the degree of erythrocyturia that may be regarded as physiological and that which is pathological has yet to be defined.

Gross haematuria is an alarming symptom for which patients seek medical advice. Severe inflammation, stones, and neoplasms are the most common causes. Gross haematuria was the presenting symptom in 91% of 3000 cases of bladder cancer (Wallace and Harris, 1965). Microhaematuria, however, has not received much attention. Among the few studies on the relationship between microhaematuria and diseases of the urinary tract only one included the possible significance of the degree of erythrocyturia (Greene et al., 1956). This apparent lack of interest may be because routine urine analysis cannot always be relied on to detect occult blood (Free et al., 1956). Nevertheless, Crabbe et al. (1956) and Booth (1959) regarded significant microscopic erythrocyturia as a useful sign in the early diagnosis of bladder cancer. Obviously if this sign is to be of value in diagnosis the method of detecting microhaematuria must be reliable and what degree of microhaematuria may be regarded as normal be known. We have screened a random population for cancer of the urinary tract by examining the cytology of the first morning urine (Freni et al., 1976; Freni and Freni-Titulaer, 1977). We found significant microhaematuria in so many cases that we decided to investigate the sensitivity of existing methods for assessing erythrocyturia.

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Subjects and methods

A POPULATION SCREENING

The trial was performed in two villages in an industrial area north of Amsterdam. Out of a total male population aged 50 years and over 432 (40.7%) participated. In addition 14 younger men were screened for various reasons. The whole volume of the unfixed first morning urine in each case was well mixed and a 40 ml sample centrifuged for 9 minutes at 2700 rpm (1230 g) in tubes with pointed bottoms. The supernatant was carefully aspirated through a Pasteur pipette, connected to a vacuum pump, and inserted just beneath the surface until about 0.5 ml fluid remained. Finally, five drops of absolute ethanol were added for initial fixation. The sediment was resuspended by pipetting and the whole suspension then transferred on to two albumen-coated slides and spread over an area of 11 cm² per slide. The slides were dried for a few minutes on a warm plate (60°C), cooled to room temperature, coated with a fixing and protecting spray (Sprayfix, Histolab, Gothenborg, Sweden), and finally stained by the Papanicolaou technique. The number of red blood cells per high-power field (RBC/hpf) were recorded as the mean of 10 readings using a × 40 dry-plane objective and × 10 wide-field binoculars. The results were classified as <1 RBC/hpf, 1-10
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RBC/hpf, 11–50 RBC/hpf, 51–100 RBC/hpf, and more than 100 RBC/hpf. In addition, the presence of white blood cells and cellular abnormalities indicative of malignancy was recorded and data on age, smoking, occupation, and possible contact with potential carcinogens were collected.

Specimens of urine from 360 participants, varying in age from 50 to 65 years, were simultaneously examined with the Hema-Combistix test strip (Ames-Miles, Elkhart, Indiana, USA) for glucose, protein, blood, and pH. The blood part of the strip is known under the trade mark of Hemastix.

B CONVENTIONAL URINE ANALYSIS
Data on erythrocyturia in 200 consecutive male outpatients not suffering from urological disease were compiled from the files of the Laboratory of Clinical Chemistry. The presence and degree of erythrocyturia were assessed by conventional routine urine analysis. After centrifuging 15 ml urine the supernatant was almost completely poured off. The last drops were shaken up and poured on to a slide to be examined wet and unstained.

C COMPARISON OF SENSITIVITY OF TESTS FOR OCCULT BLOOD
A stock blood suspension was made by adding 1·0 ml blood (Hb 95%; RBC about 5 × 10⁶/mm³) to 1 litre urine, pH 5·5, which had been centrifuged and filtered. Final dilution samples were examined simultaneously by the following methods: (1) centrifugation and microscopy of stained slides as described in investigation A; (2) conventional analysis as described above in B, but the tests were performed by a trained cytotechnician in the cytology laboratory; (3) using a reagent strip of commercially available Hemastix, the appearance of a blue colour within 30 seconds indicating the presence of blood; (4) using a recent modification of Hemastix not then commercially available; and (5) using a reagent strip of Sangur-Test (Boehringer, Mannheim, Germany); the appearance of green spots within 60 seconds indicating the presence of blood: under a magnifying glass a seemingly negative test strip might show minute green spots representing single erythrocytes.

Results
The incidence and degree of microscopic haematuria in apparently healthy men (investigations A and B) are summarised in Table 1 together with the findings of other investigators. Not one urine was free of erythrocytes. More than one RBC/hpf was found in 19·3% of cases. In 33 cases the cytological picture was compatible with 'pyelocystitis'. In five cases there were cellular abnormalities suggestive of a tumour. These five patients and all those with erythrocyturia exceeding 50 RBC/hpf were sent for full urological examination. The clinical findings in all the patients after a sufficiently long follow-up will be published in due course.

Preliminary findings include one case of borderline carcinoma-in-situ of the bladder, two cases of renal stones, and three cases of severe prostatic hyperplasia with obstruction. The results of routine conventional urine analysis performed in a routine clinical laboratory (investigation B) were clearly inferior to those with the same technique performed by a trained cytotechnician (investigation C 2), as may be concluded from Tables 1 and 2. Commercially available Hemastix gave positive results in only 2 out of 360 samples (investigation A), and one of these was probably a false positive. In this case cytological examination revealed only 25 RBC/hpf, or about 15 000 RBC/ml, which is far beyond the claimed sensitivity.

The results of the sensitivity tests in investigation C are shown in Table 2. The cytological method seemed the most sensitive but also the most time-consuming for detecting occult blood. The Sangur-Test was the most sensitive test strip, especially when read with a magnifying glass.

Discussion
Erythrocytes were found by cytological examination in all the samples of urine. In 8·3% of cases more than 10 RBC/hpf were present. This is in contrast to Wright's finding (1959) that only 21·6% of 5000 healthy men had erythrocytes in their urine and only 2% had more than 10 RBC/hpf. Sanders (1963) found much higher incidences in 730 male laboratory workers (Table 1). He carefully removed the supernatant and applied suction for the last millilitre, while Wright simply poured off the liquid. Rofe (1955) found blood in all his samples, most probably owing to his method of concentration and use of benzidine to stain RBC specifically. On the other hand, Rofe did not find a daily loss of more than 312 000 RBC (about 250 RBC/ml urine) in a total of 12 men. This degree of erythrocyturia roughly corresponds to one RBC/hpf by our technique. This very low number and the small number of men examined make Rofe's results difficult to interpret.

The acceleration of gravity (g) applied in centrifuging was not mentioned in any of the papers we studied except Rofe's (1955). In many laboratories, however, a centrifuge with tubes at a fixed angle is used. The resulting sedimentation force is lower than might be assumed from the revolutions per minute. Moreover, the cells are sedimented over a larger area, which greatly increases the risk of cell loss.
when the supernatant liquid is discarded. According to Gadeholt (1964) the number of cells per microscopic field varied from N to 10N when the sediment was transferred by pouring and from N to 3N when a Pasteur pipette was used. He thought that centrifugation was responsible for a cell loss of more than 50%, while considerable loss might occur if no precautions were taken to minimise haemolysis.

We decided to centrifuge 40 ml urine instead of 15 ml to get higher total yields of epithelial cells and thus increase the chance of detecting cancer cells. The number of cells per hpf, however, could even be lower than in a wet slide of one or two drops, since the sediment is distributed over 22 cm² while a wet preparation covers about 6 cm². We did not intend to recover all the cellular content since this is impracticable in a mass cytological screening for which the method was developed. But we took great care to obtain reproducible results by standardising the methods of centrifugation, collection of sediments, and preparation of smears. Precautions were taken to minimise cell loss in the supernatant, loss of cells adhering to the tube wall, and false counting caused by cell clumping or misinterpretation of cell types. Erythrocytes are easily recognised in stained slides and screening for cancer cells can be performed simultaneously.

Several authors have doubted the adequacy of centrifugation compared with test strips (Leonards, 1962; Longfield et al., 1962; Kutter et al., 1974; Braun and Straube, 1975). Hemastix is said to detect blood in a dilution of 1:500 000 in water (Product Information, Ames, 1964), which is equal to about 10 000 RBC/ml. The strip is said to be less sensitive in urine because of the presence of inhibitors. Since these may vary in nature and concentration from specimen to specimen it is impossible to give a definite figure for the sensitivity of Hemastix strips to blood in urine. Leonards (1962) and Welschbellig (1964) reported that these strips can detect blood in urine in a dilution of 1:50 000 (about 100 000 RBC/ml urine). The results with two different batches of commercial Hemastix in our investigation A, however, were very disappointing. Investigation C confirmed that the strip is unacceptably insensitive. A concentration of 1 000 000 RBC/ml, which is found to be the lower limit (Table 2), can hardly be considered microhaematuria. The new, modified Hemastix, however, was much more sensitive. Positive reactions were constantly obtained in a maximum blood dilution of 1:100 000, equal to 50 000 RBC/ml urine. The sensitivity found

### Table 1  Incidence and degree of microscopic haematuria in apparently healthy males (investigations A and B)

<table>
<thead>
<tr>
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<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cytology No. (%)</td>
<td>Analysis¹</td>
<td>Analysis No. (%)</td>
<td>Occulttest No. (%)</td>
<td>Analysis No. (%)</td>
</tr>
<tr>
<td>No blood</td>
<td>-</td>
<td>127 (63.5)</td>
<td>24 (48)</td>
<td>50 (100)</td>
<td>344 (89.1)</td>
</tr>
<tr>
<td>Occasional</td>
<td>360 (80.7)</td>
<td>55 (27.5)</td>
<td>22 (44)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1-10</td>
<td>49 (11.0)</td>
<td>16 (8.0)</td>
<td>4 (8)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>11-50</td>
<td>24 (5.4)</td>
<td>2 (1.0)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>51-100</td>
<td>5 (1.1)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>&gt; 100</td>
<td>5 (1.1)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Total</td>
<td>446</td>
<td>200</td>
<td>50</td>
<td>386</td>
<td>5000</td>
</tr>
</tbody>
</table>

¹Urinary analysis performed in the clinical chemistry laboratory (investigation B).

### Table 2  Microscopic haematuria as assessed by different techniques (investigation C)

<table>
<thead>
<tr>
<th>Blood dilution in urine</th>
<th>Real content (RBC/ml)</th>
<th>Analysis (RBC/hpf)¹</th>
<th>Cytology (RBC/hpf)</th>
<th>Hemastix</th>
<th>New Hemastix</th>
<th>Sangur-Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:5000</td>
<td>1 000 000</td>
<td>&gt; 50</td>
<td>&gt; 50</td>
<td>Trace/ +</td>
<td>+ + +</td>
<td>+ + +</td>
</tr>
<tr>
<td>1:10 000</td>
<td>500 000</td>
<td>&gt; 50</td>
<td>&gt; 50</td>
<td>-</td>
<td>+ + +</td>
<td>+ + +</td>
</tr>
<tr>
<td>1:25 000</td>
<td>200 000</td>
<td>35-50</td>
<td>200-500</td>
<td>-</td>
<td>+ + +</td>
<td>+ + +</td>
</tr>
<tr>
<td>1:50 000</td>
<td>100 000</td>
<td>24-28</td>
<td>100-200</td>
<td>-</td>
<td>+ + +</td>
<td>+ + +</td>
</tr>
<tr>
<td>1:100 000</td>
<td>50 000</td>
<td>12-14</td>
<td>68-110</td>
<td>-</td>
<td>+ + +</td>
<td>+ + +</td>
</tr>
<tr>
<td>1:250 000</td>
<td>20 000</td>
<td>6-10</td>
<td>32-60</td>
<td>-</td>
<td>+ + +</td>
<td>+ + +</td>
</tr>
<tr>
<td>1:500 000</td>
<td>10 000</td>
<td>4-7</td>
<td>11-24</td>
<td>-</td>
<td>-</td>
<td>Trace²</td>
</tr>
<tr>
<td>1:1 000 000</td>
<td>5000</td>
<td>2-4</td>
<td>4</td>
<td>-</td>
<td>-</td>
<td>Trace²</td>
</tr>
<tr>
<td>1:10 000 000</td>
<td>500</td>
<td>&lt; 1</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1:100 000 000</td>
<td>50</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

¹Urinary analysis performed in the cytology laboratory.
²Test strip read with magnifying glass.
for Sangur-Test (Table 2) more or less matched those reported by Kutter et al. (1974) and Braun and Straube (1975), who had positive responses in a minimum concentration of 1000 RBC/ml. In our experience, however, such a low concentration will only occasionally give one single green spot on the test strip. In routine practice this would be scored negative. Definite positive scores were obtained at about 5000 RBC/ml, but only when a magnifying glass was used for reading the strip. At concentrations of 20000 or more RBC/ml the number of green spots is enough to be easily seen by the naked eye.

A possible explanation for the discrepancy between our results with the original Hemastix and those of Leonards (1962) and Welschbillig (1964) might be the method of preparing the dilution samples. We initially prepared the stock suspension by adding 0.2 ml blood to 1 litre saline and found the sensitivity limit at 100 000 RBC/ml. The sensitivity decreased progressively with less saline in the samples. In investigation C saline was omitted, since stocks containing saline lead to final test samples with correspondingly decreased amounts of inhibiting factors present in urine, and thus to falsely high sensitivity values. Leonards did not mention how his dilution samples were made, but Welschbillig used stock suspensions based on saline. We found that Sangur-Test reactions were not influenced when urine was replaced by urine-saline mixtures.

Another cause for falsely high sensitivities is the use of uncentrifuged urine of 'healthy' volunteers for preparing the dilution samples. Our findings show that no urine can be presumed to be free from blood. More than 500 RBC/ml might be present in the urine of 20% of healthy males and even more than 7000 RBC/ml in 8%. These percentages may be even higher in control groups composed of hospital patients. Hence the results with any sensitivity test based on samples diluted with uncentrifuged urine, even if obtained from 'healthy young men' (Braun and Straube, 1975), are incorrect since such samples contain many more erythrocytes than calculated.

Efforts to increase the sensitivity of tests for detecting blood are based on the assumption that there is a physiological degree of microhaematuria clearly demarcated from higher, abnormal values. But the clinical significance of microhaematuria is still debatable. Hypertension, asymptomatic hyperplasia of the prostate, and cystitis, for example, might be associated with microhaematuria (Greene et al., 1956; Higgins, 1958; Tschan et al., 1975). These diseases are common in older people as is erythrocyturia of less than 20 000 RBC/ml, and their simultaneous occurrence might therefore be coincidental. Moreover, with a causal relationship a correlation between the degree of erythrocyturia and the extent of the lesion would be expected. We found only one report on the clinical significance of microhaematuria which included its degree. Greene et al. (1956) remarkably concluded from this study that they 'failed to disclose any factors that permit the urologist to differentiate microhaematuria of significant origin from that of insignificant origin... The grade of microhaematuria is not decisive.'

Possibly Greene's results, based on conventional urine analysis, were greatly influenced by inaccuracy in their technique. Analysis of 200 urinary samples from a hospital population, performed in a routine clinical laboratory (our investigation B), showed that only one per cent of the cases had more than 10 RBC/hpf. That differs greatly from the 8.3% found by cytology in a healthy non-hospital population (Table 1). The results of our investigations C1 and C2, however, made it clear that conventional analysis, performed by a cytotechnician trained in qualitative methods, might be almost as sensitive as the cytology technique (Table 2). It is unfortunate that conventional urine analysis looks so simple and that it is usually left to the most junior technician to perform.

Kutter et al. (1974) and Braun and Straube (1975) summarised the available reports with their statement that 5000 RBC/ml is the upper limit for physiological microhaematuria. They overlooked, however, that they reviewed results that were based on conventional urine analysis, a technique criticised by themselves for its inaccuracy. Judged purely statistically, the results of our population survey of less than 20 000 RBC/ml urine is most probably an insignificant figure, since it comprised about 95% of the population studied. Since no clear influence of age and occupation on the degree of microhaematuria was found (Freni et al., 1977), this population might be regarded as more or less representative for any healthy male population.

We would therefore tentatively suggest that the line between physiological and abnormal values, if it exists at all, should be drawn at a level of about 20 000 RBC/ml. The corresponding value in conventional urine analysis as performed in our laboratory is about 8 RBC/hpf and in our cytology technique about 40 RBC/hpf. Thus both the new Hemastix and Sangur-Test seem suitable for detecting possible abnormal degrees of microhaematuria. The real value of these test strips, however, has still be confirmed in critical, large-scale studies. These should include a full urological examination of the strip-positive cases and a follow-up of the strip-negative cases.
References


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