



# DIRECT-IN-TUBE RAPID PAPANICOLAOU STAINING (PAPR) FOR SEROUS EFFUSIONS: CYTOMORPHOLOGICAL AND DIAGNOSTIC PERFORMANCE EVALUATION

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## ABSTRACT

**Background.** Serous effusion cytology is essential for evaluating inflammatory and neoplastic processes. Conventional Papanicolaou staining, although considered the reference standard in cytopathology, requires multiple processing steps and substantial reagent consumption.

**Objective.** To validate a rapid modified Papanicolaou staining method applied directly in tube (PapR) for serous effusions and compare its diagnostic and operational performance with the conventional technique.

**Materials and Methods.** Eighty serous effusion samples (28 pleural, 26 peritoneal, 26 pericardial) were processed in parallel using conventional Papanicolaou staining (PAP) and PapR. Cytological interpretation followed TIS 2020 guidelines. Concordance analysis, Cohen's Kappa coefficient, and diagnostic performance parameters were calculated. Slide adequacy was assessed using a structured Degree of Suitability for Interpretation (DSI) score.

**Results.** Global diagnostic concordance was 97.5% ( $\kappa = 0.95$ ,  $p < 0.001$ ) for the five-category TIS classification. When AUS and SFM categories were grouped as cytologically positive for atypia, PapR demonstrated 100% sensitivity and 98.1% specificity, with a false-positive rate of 1.25% and no false-negative cases identified. Two cases were discordant between methods: one case initially classified as NFM on conventional PAP was reclassified as SFM on PapR (single false-positive result), while the second discordance represented a minor categorical variation that did not modify the positive/negative diagnostic grouping. Cytomorphological features were preserved, and approximately 71% of cellular sediment remained available after staining. When analyzed using binary grouping (negative vs positive for atypia), concordance increased to 98.75% (79/80 cases).

**Conclusion.** PapR represents a rapid, reliable, and resource-efficient alternative to conventional Papanicolaou staining for serous effusions, demonstrating excellent diagnostic performance while optimizing laboratory workflow and preserving biological material.

**Keywords:** Papanicolaou stain, serous effusions, cytopathology, rapid staining, TIS 2020

## Introduction

Serous effusions – pleural, peritoneal, and pericardial – represent a highly valuable biological material in cytopathological diagnostics, commonly used to identify inflammatory, reactive, or neoplastic processes. Cytological analysis of these fluids plays a crucial role in cancer staging, prognosis evaluation, and monitoring treatment response, offering a

minimally invasive alternative to exploratory surgical procedures (1–3).

Conventional Papanicolaou staining, although considered the reference standard in cytopathology, was originally developed for gynecologic smears but has been widely applied to serous fluid cytology due to its ability to highlight fine nuclear details, the nuclear-to-cytoplasmic ratio, and chromatin patterns. These features are essential for differentiating reactive

mesothelial cells from neoplastic cells (4–7). Its efficiency, reproducibility, and relatively low cost have established Papanicolaou staining as a reference standard in cytopathology.

However, conventional processing involves multiple sequential steps, including fixation, hydration, nuclear and cytoplasmic staining, differentiation, dehydration, and clearing. Each step requires defined exposure times and significant volumes of reagents. In high-volume laboratories, this approach can be time-consuming, prone to artifacts (air-drying, uneven staining, cross-contamination), and may result in cellular material loss.

Over recent decades, several modified Papanicolaou staining protocols have been proposed to reduce processing time and reagent use without compromising morphological quality. These adaptations primarily focused on shortening dye exposure times, reducing the number of alcohol baths, and implementing rapid fixation protocols (8-12).

In this context, developing a staining method applied directly in the tube on the cellular sediment obtained from serous effusions offers a viable solution for workflow optimization in cytopathology laboratories. This approach has the potential to decrease total processing time, minimize reagent consumption, and reduce artifact formation, while maintaining the diagnostic performance required for accurate cytological assessment.

The aim of the present study was to evaluate and validate a rapid Papanicolaou staining method applied directly in the tube (PapR) for pleural, peritoneal, and pericardial effusions by comparing it with the conventional technique in terms of cytomorphological, chromatic, quantitative, and statistical performance. The primary endpoint was to assess the ability of the PapR method to detect cytological atypia according to TIS 2020 criteria, while preserving morphological quality and diagnostic reproducibility (13-15).

**Materials and methods**

The present study was designed as an experimental, prospective, comparative investigation conducted throughout 2025 at the Center for Research and Development for

**Morphological and Genetic Studies of Malignant Pathology (CEDMOG).**

Biological samples were obtained from patients admitted to the Clinical Emergency County Hospital Constanța, from outpatient clinics, and from affiliated medical networks. All samples were submitted to the institution accompanied by documented informed patient consent. (Table 1).

*Table.1 Distribution according to anatomical site was as follows:*

| Type of Effusion | Number of Samples | Percentage (%) |
|------------------|-------------------|----------------|
| Pleural          | 28                | 35             |
| Peritoneal       | 26                | 32.5           |
| Pericardial      | 26                | 32.5           |
| Total            | 80                | 100            |

The study included all serous effusion samples that fulfilled institutional quality criteria for collection and processing, as defined by the Technical Competency and Standard Procedures Manual and the Standard Operating Procedures (SOP) Manual of the institution.

Preanalytical considerations recommended by international guidelines applicable to serous fluid cytology were strictly respected to ensure adequacy of the biological material and preservation of diagnostic accuracy.

Detailed data collection procedures and laboratory processing steps are described below.

**Study Objective**

The objective of the study was to evaluate the cytomorphological and diagnostic performance of a rapid modified Papanicolaou staining method applied directly in the tube (PapR), compared with the conventional Papanicolaou technique (PAP) performed on classical cytological smears.

The protocol was designed to allow parallel analysis of the same biological samples using both staining methods, thereby ensuring direct comparability of results and minimizing inter-sample variability.

**Study Population and Sample Distribution**

A total of 80 serous effusion samples were included during the study period. Samples originated from hospitalized or ambulatory

patients.

All samples were collected, labeled, numbered, and registered according to standard laboratory procedures. Transport conditions were controlled to prevent cellular degradation prior to processing.

#### **Inclusion Criteria**

- Sufficient serous fluid volume to allow dual processing
- Absence of macroscopic coagulation
- Sample integrity at the time of laboratory reception

#### **Exclusion Criteria**

- Insufficient volume
- Severe hemolysis
- Advanced cellular degradation likely to compromise cytological quality

#### **Sample Processing**

Upon laboratory reception, each specimen was gently homogenized and subjected to standardized centrifugation to obtain the cellular sediment.

The supernatant was carefully removed, and the sediment was resuspended in a controlled volume of cytological fixative based on 96% ethyl alcohol to ensure uniform cellular distribution.

The cellular sediment was subsequently divided into two equal fractions: one fraction processed using the conventional Papanicolaou technique (PAP) and one fraction processed using the rapid PapR method applied directly in the tube.

This paired-sample design enabled direct comparative evaluation of the two techniques on identical biological material.

Conventional Papanicolaou Staining (PAP) - for the conventional method, the cellular sediment was used to prepare cytological smears manually spread onto clean, degreased glass slides.

Smears were immediately fixed in 96% ethyl alcohol to prevent air-drying artifacts and subsequently subjected to the standard Papanicolaou staining protocol..

The staining process included the classic steps of hydration, nuclear staining with hematoxylin, differentiation, cytoplasmic staining with Orange G and Eosin Azure, followed by progressive dehydration, clarification and final mounting. The total duration of conventional

processing required multiple transfers of the slides to the dye baths and alcohol solutions.

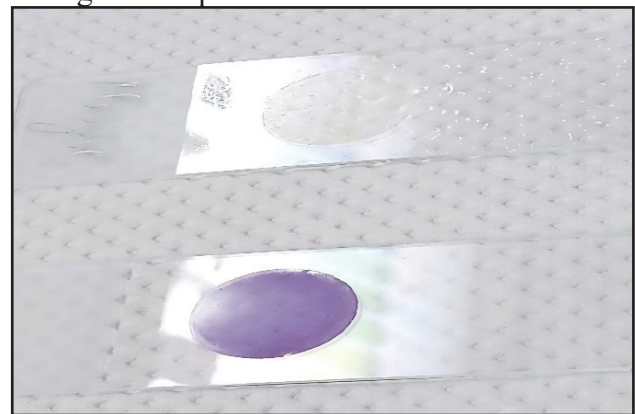
The conventional protocol required multiple slide transfers through staining baths and alcohol solutions.

#### **Rapid Papanicolaou Staining in Tube (PapR)**

The PapR method consisted of direct application of Papanicolaou reagents to the cellular sediment within the centrifuge tube, without initial smear preparation. Following centrifugation, the sediment underwent sequential steps of fixation, nuclear staining, and cytoplasmic staining, using reduced exposure times optimized to preserve cytomorphological quality.

After completion of staining, the colored sediment was used to prepare cytological slides using a liquid-based cytology approach, resulting in monolayer preparations with uniform cellular distribution and reduced cellular overlap (Figure 1).

This technique allowed efficient utilization of available cellular material and enabled preparation of multiple slides from the same biological sample.



*Figure 1. Smears spread out in monolayer, obtained by the two methods compared. Upper panels: unsustained smears (post-centrifugation) for conventional staining. Lower panels: pre-stained smears (prior to centrifugation) using PapR*

#### **Cytomorphological Evaluation**

Slides obtained by both methods were evaluated independently using qualitative and quantitative criteria, including: cellularity, preservation of nuclear details (nuclear contour, chromatin pattern, nucleoli), preservation of cytoplasmic characteristics, nuclear-to-

cytoplasmic ratio, degree of hemorrhagic or proteinaceous background, presence of artifacts, overall slide readability. To allow numerical quantification of qualitative cytological evaluation, the Degree of Suitability for Interpretation (DSI) was calculated prior to statistical analysis. The DSI score ranged from 1 to 5, with higher values indicating better suitability for interpretation. DSI values were used as quantitative variables in comparative statistical analyses.

Cytological interpretation was performed according to The International System for Reporting Serous Fluid Cytopathology (TIS 2020), with classification into five diagnostic categories: ND – Non-diagnostic, NFM – Negative for Malignancy, AUS – Atypia of Undetermined Significance, SFM – Suspicious for Malignancy, MAL – Malignant

#### Statistical Analysis

Data were initially recorded in Microsoft Excel and subsequently exported to IBM SPSS Statistical Analysis Software, Release 30.0.0. The conventional PAP method was considered the reference standard.

Diagnostic performance parameters calculated for PapR included: sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), overall diagnostic accuracy.

For performance analysis, AUS and SFM categories were grouped as cytologically positive results. Binary classification was defined as follows: ND and NFM as negative; AUS and SFM as positive.

Diagnostic concordance between methods was evaluated using paired data derived from identical biological samples. Statistical association was assessed using the Chi-square test. Concordance strength was measured using Cohen's Kappa coefficient ( $\kappa$ ), interpreted according to Landis and Koch criteria. Results were interpreted in the context of diagnostic performance, reproducibility, and operational laboratory impact.

#### Specimen Allocation and Material Preservation Strategy

To optimize biological material utilization, a standardized specimen allocation protocol was applied. When additional cytochemical stains

(e.g., Giemsa) were required as part of routine cytopathological evaluation, the sediment was divided into two separate aliquots prior to processing. This ensured adequate material for both conventional smear preparation and complementary staining techniques.

Moreover, the residual sediment remaining after smear preparation was incorporated into the cell block whenever feasible. This approach allowed preservation of cellular material for potential ancillary studies, including special stains and immunohistochemical investigations. Such pre-analytical planning minimized material loss and enhanced the overall diagnostic yield, particularly in low-cellularity effusions.

## Results and Discussion

### Cytomorphological Quality

In the majority of cases, the PapR method produced preparations with sufficient cellularity, uniform monolayer distribution, and a relatively clean background, with reduced cellular overlap and fewer mechanical artifacts.

Qualitative analysis demonstrated that PapR preserved essential nuclear and cytoplasmic features required for cytological diagnosis, including nuclear contour, chromatin characteristics, nucleoli, and nuclear-to-cytoplasmic ratio.

No significant differences affecting diagnostic interpretation were observed compared with the conventional PAP method (Table 2).

*Table 2. DSI – Degree of Suitability for Interpretation, score from 1 to 5.*

| Staining Method  | Mean DSI | Adequate Slides (%) | Inadequate Slides (%) |
|------------------|----------|---------------------|-----------------------|
| Conventional PAP | 4.6/5    | 95                  | 5                     |
| PapR             | 4.5/5    | 93                  | 7                     |

The background in PapR preparations showed reduction of hemorrhagic and proteinaceous material in numerous cases, facilitating identification of relevant cellular elements, particularly in low-cellularity samples (Figure 2, objective magnification  $\times 20$ ).

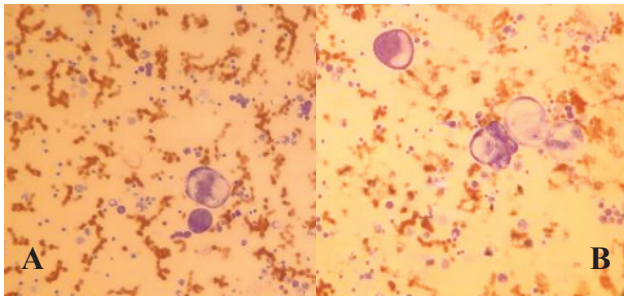


Figure 2. The hemorrhagic sample, suggestive of malignancy, a) col. PAP and b) col. PapR, ob. 20x.

### Final Cytological Interpretation According to TIS 2020

The majority of samples were classified as NFM. No cases were categorized as MAL. Seventy-eight out of 80 cases were classified identically by both methods, yielding a global concordance rate of 97.5% for the five-category classification.

Two cases were discordant between the two methods. One case initially classified as NFM on conventional PAP was reclassified as SFM on PapR (single false-positive result). The second discordant case represented a minor reclassification within TIS categories without changing the overall binary grouping. When analyzed using binary grouping, concordance increased to 98.75% (79/80 cases) (Table 3).

Table 3. Distribution of diagnostic categories (n = 80)

| TIS Category | PAP (n) | PAP (%) | PapR (n) | PapR (%) | Concordance (%) |
|--------------|---------|---------|----------|----------|-----------------|
| ND           | 5       | 6.25    | 5        | 6.25     | 100             |
| NFM          | 53      | 66.25   | 52       | 65.00    | 98.1            |
| AUS          | 10      | 12.5    | 10       | 12.5     | 100             |
| SFM          | 12      | 15.0    | 13       | 16.25    | 91.7            |
| MAL          | 0       | 0       | 0        | 0        | –               |
| Total        | 80      | 100     | 80       | 100      | 97.5            |

Table 4. Diagnostic Performance of PapR

| Parameter                       | Value (%) |
|---------------------------------|-----------|
| Sensitivity                     | 100       |
| Specificity                     | 98.1      |
| Positive Predictive Value (PPV) | 95.7      |
| Negative Predictive Value (NPV) | 100       |
| False-negative rate             | 0         |
| False-positive rate             | 1.25      |
| Cohen's Kappa                   | 0.95      |
| p-value (Chi-square)            | < 0.001   |

When AUS and SFM categories were grouped as cytologically positive results, PapR demonstrated 100% sensitivity and 98.1% specificity compared with the conventional PAP method used as reference standard.

The absence of false-negative results indicates that PapR did not miss any atypical or suspicious cases identified by conventional PAP.

The single false-positive case represented an upgrade from NFM to SFM, without clinical impact. The second discordant case did not modify the positive/negative diagnostic grouping and therefore did not affect calculated sensitivity or specificity.

Chi-square analysis demonstrated a statistically significant association between methods ( $p < 0.001$ ). Cohen's Kappa coefficient ( $\kappa = 0.95$ ) indicated almost perfect agreement according to Landis and Koch criteria.

These findings confirm that the rapid PapR method maintains diagnostic reliability comparable to the conventional technique while offering important operational advantages.

### Residual Cellular Sediment Analysis

A relevant operational finding was the amount of residual cellular sediment remaining after PapR processing. Quantitative analysis showed that approximately 71% of the initial sediment volume remained available after direct tube staining. Mean sediment loss was below 29%, without affecting morphological evaluation quality.

Additionally, residual sediment remained morphologically stable for up to 7 days at 4°C, allowing reanalysis or supplementary investigations if necessary (Figure 3).



Figure 3. Sample evaluated before (a) and after (b) PapR staining.

## Operational Advantages

Compared with the conventional method, PapR allows:

- More efficient utilization of cellular material
- Increased number of potential slides from the same sample
- Reduced biological material loss
- Lower reagent consumption
- Optimization of laboratory workflow

These operational advantages support implementation of PapR in high-volume cytopathology laboratories where rapidity, reproducibility, and rational resource utilization are essential.

Furthermore, the residual sediment remaining after smear preparation may be incorporated into the cell block. This strategy allows preservation of biological material for subsequent ancillary investigations, including special stains or immunohistochemical studies. Such an integrated workflow maximizes diagnostic yield while maintaining efficient use of limited specimens, particularly in low-cellularity effusions.

## Conclusion

PapR demonstrates excellent diagnostic performance, showing 100% sensitivity and 98.1% specificity, maintaining very high concordance with conventional PAP evaluation (97.5% for five-category classification and 98.75% for binary grouping,  $\kappa = 0.95$ ).

The single false-positive reclassification from NFM to SFM did not affect clinical management, and no false-negative cases were observed. PapR preserves cytomorphological quality while significantly improving laboratory workflow efficiency and biological material utilization.

These findings support PapR as a reliable and safe alternative to conventional Papanicolaou staining in the evaluation of serous effusions.

**Disclosure of conflict of interest:** None to declare.

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