Monique Fabre, MD

- Consultant Cyto-and Histopathologist, Necker-Enfants Malades University Hospital, Paris.
- Associate Professor of Pathology
- Past-President of the French Society of Clinical Cytology.
- Centres of interest: Quality Control in Cytopathology, Deep Organs FNA, Paediatric, Pancreas, Liver, and Digestive Pathology.
- Topic: Papanicolaou staining of cytology specimens: best practice recommendations Up-to-date information from the first French External Quality Assurance survey.
Papanicolaou Staining of Cytology Specimens

Good Practice Recommendations
Up-to-date information from the first French External Quality Assurance Survey on Gyn Cytology
Preliminary results

Monique Fabre, Necker Enfants-Malades University Hospital, Paris

No conflict of interest
The polychrome Papanicolaou (Pap) staining method has gained worldwide acceptance for cytologic samples.
Principles of Pap staining

• Multichromatic staining technique
• Classic form: five dyes in three solutions
• Fixation:
  – Smear should be quickly fixed
  – Alcohol gives better nuclear detail than air
• Staining
  – Blue green for ribosomes, particularly in parabasal cells and metaplastic squamous cells
  – Pink in inactive cells, such as superficial cells
  – Orange in keratinized cells
External Quality Assessment (EQA)

• Maintain and improve the quality of patient care by promoting a high standard of performance.
• The staining of cervical samples by Pap technique is an integral part of the screening process. Any failure or deterioration in this staining procedure may deliver sub-standard results.
• This scheme applies equally to conventional smears and LBC preparations.
Objectives of EQA

- Provide an external assessment of Pap staining quality in cervical samples.
- Establish minimum quality requirements for staining.
- Track substandard stains, determine causes, and define corrective actions.
- Promote quality by developing consistent good practice.
The French Experience:

Association Française d’Assurance Qualité en Anatomie et Cytologie Pathologiques

External quality assurance organisation:

– Diagnostics

– Laboratory techniques

  • Histology stains: HE, PAS, Reticulin, ....

  • IHC tests

  • Cytology stains: MGG in 2013, Papanicolaou/Harris-Shorr in 2015

– Quality control of molecular diagnostic platforms.

Laboratories participate on a voluntary basis and with respect of anonymity.
Conditions to participate to the test

• One test by Institution/Lab
• Send 2 home-stained trophic Gyn slides (with exo- and endocervical cells) to AFAQAP
• By February 2016
• With
  – 2 types of permitted preparation
    • Conventional smearing
    • Or LBC
  – 2 types of permitted staining
    • Pap
    • Or Harris-Shorr
• And fill an online questionnaire in parallel
Questionnaire content

• **Staining protocol details (48 issues)**
  – types of liquid-based solution
  – automated procedures
  – dyes suppliers
  – reagents
  – mounting
  – HPV genotyping

• **General information (34 issues)**
  – public/private hospital or office
  – number of exams/month
  – number of cytotechnologists, pathologists
  – training
Methods for test evaluation (1)

June 2016, 1st jury meeting (one full day)

- 8 SFCC assessors, using a multiheaded microscope
- Score sheet: review and validation (32 pretest cases)
- The assessment score sheet (on a maximum scale of 30) was calibrated on excellence.
- For an average or good score, the smears were of sufficient quality to allow analysis
- Five main parameters:
  - overall quality of preparation and mounting (1-2)
  - nuclear staining of squamous cells: chromatin, hematoxylin color, differentiation (1-12)
  - cytoplasmic staining of squamous cells: colour spectrum, intensity of cyanophilia, eosino/orangeophilia (1-12)
  - nuclear staining of endocervical cells/metaplastic cells (0-2)
  - cytoplasmic staining of endocervical cells/metaplastic cells (0-2)
## Score Sheet for the Assessment of PAP/Harris-Shorr Staining (1)

The assessment was calibrated on excellence

<table>
<thead>
<tr>
<th>Assessment of the overall quality of preparation and mounting</th>
<th>1-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Translucency of the slide, optimal dispersion of the cells</td>
<td>2</td>
</tr>
<tr>
<td>Poor preparation of slide: air bubbles, carbowax/dye deposit, &quot;corn flakes&quot; nuclei, hole/thick fragments/poor cell spreading</td>
<td>1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Assessment of nuclear staining of squamous cells</th>
<th>1-12</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Chromatin</strong></td>
<td>1-4</td>
</tr>
<tr>
<td>Crisp, granular and distinct pattern in virtually all nuclei and sharp contrast</td>
<td>4</td>
</tr>
<tr>
<td>Crisp and distinct chromatin pattern in the majority of nuclei</td>
<td>3</td>
</tr>
<tr>
<td>Chromatin visible, but lacking definition, in the minority of nuclei</td>
<td>2</td>
</tr>
<tr>
<td>Chromatin visible, but lacking definition, in the majority of nuclei</td>
<td>1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Hematoxylin color</strong></th>
<th>1-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blue/black colour in virtually all nuclei, without adversely affecting the colours of the counterstains</td>
<td>4</td>
</tr>
<tr>
<td>Blue/black colour in the majority of nuclei</td>
<td>3</td>
</tr>
<tr>
<td>Pink/red/green colour in more than 50% of nuclei</td>
<td>2</td>
</tr>
<tr>
<td>Pink/red/green colour in virtually all nuclei</td>
<td>1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Differentiation</strong></th>
<th>1-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Optimal intensity of nuclear staining in virtually all nuclei</td>
<td>4</td>
</tr>
<tr>
<td>Acceptable intensity of nuclear staining</td>
<td>3</td>
</tr>
<tr>
<td>Nuclei overstained and affecting cytoplasm</td>
<td>2</td>
</tr>
<tr>
<td>All nuclei heavily overstained, with haematoxylin in cytoplasm throughout</td>
<td>1</td>
</tr>
</tbody>
</table>
# Score Sheet for the Assessment Of PAP/Harris-Shorr Staining (2)

## Assessment of cytoplasmic staining of squamous cells (take into account the hormonal status) 1-12

<table>
<thead>
<tr>
<th>Colour spectrum</th>
<th>1-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Optimal egal intensity of cytoplasmic staining: superficial pink, less mature blue/green, keratinised orange/yellow</td>
<td>4</td>
</tr>
<tr>
<td>Good intensity of cytoplasmic staining throughout the slide</td>
<td>3</td>
</tr>
<tr>
<td>Acceptable intensity of cytoplasmic staining throughout the slide</td>
<td>2</td>
</tr>
<tr>
<td>Inappropriate overall intensity, ie eosinophilia/orangeophilia virtually absent</td>
<td>1</td>
</tr>
</tbody>
</table>

## Intensity of cyanophilia 1-4

| Optimal intensity of cytoplasmic staining throughout the slide | 4 |
| Good intensity of cytoplasmic staining throughout the slide | 3 |
| Acceptable intensity of cytoplasmic staining throughout the slide | 2 |
| Inappropriate overall intensity, | 1 |

## Intensity of eosino/orangeophilia 1-4

| Optimal intensity of cytoplasmic staining throughout the slide | 4 |
| All three colours present, but one or more is underrepresented in the minority of the slide | 3 |
| One or more colours is grossly underrepresented or absent in the majority of the slide | 2 |
| All green, all pink, all orange or two tones only | 1 |

## Assessment of nuclear staining of endocervical cells and metaplastic cells if present 0-2

| Optimal intensity of nuclear staining in virtually all nuclei, some with purplish nucleoli | 2 |
| Poor nuclear staining | 1 |
| Absence of endocervical cells | 0 |

## Assessment of cytoplasmic staining of endocervical cells and metaplastic cells if present 0-2

| Optimal intensity of cytoplasm: columnar cells, terminal bars, pink cilia, vacuoles, cloudy/translucent cytoplasm | 2 |
| Poor cytoplasmic staining | 1 |
| Absence of endocervical cells | 0 |
Methods for test evaluation (2)

September 2016, 2d jury meeting (one full day)

• 7 SFCC assessors
• Each slide assessed over a multiheaded microscope
• Calculation of the mean for each slide
• If difference more than 10% between 2 assessors, a second scoring was performed in order to reach a consensus agreement.
Preliminary Results: 51 institutions

2 Harris-Shorr Gyn Tests
- 1 Conventional
- 1 LBC (Hologic)

49 Papanicolaou Gyn Tests
- 8 Conventional
- 41 LBC

Bar chart showing the distribution of tests across different brands:
- Alphapath: 1
- VWR: 3
- Novacyt: 6
- Becton Dickinson: 10
- Hologic: 21
- Total: 41

Hologic and Becton Dickinson categories are further broken down by Prepstain, Totalys, Prepmate+manuel, TP 2000, and TP5000.
Preliminary statistical analysis

All Techniques (Pap and Harris-Shorr)

- **Very Good**: 1
- **Average/Good**: 28
- **Below average**: 22
- **Poor**: 0

4 classes:
- ≥ 25 Very good
- 18 < 25 Average/Good
- 6 < 18 Below average
- < 6 Poor

Bar charts comparing liquid-based and conventional methods.
Results for the different types of LBC solutions n=42

One Harris-Shorr staining with a below average scoring

- ThinPrep: 10 Below average, 12 Average/Good or Very good
- SurePath: 3 Below average, 7 Average/Good or Very good
- Others LBC*: 8 Below average, 2 Average/Good or Very good

*Others LBC: Cyt-All® (Alphapath) n=1, EasyFix® (VWR) n=3, NovaPrep® (Novacyt) n=6
Distribution of Labs in France

- Total: 330
- Private: 190
- Public: 140

Similar distribution of Labs for this EQA

- Total: 49
- Private Labs: 26
- Public Labs: 23

Similar results for private and public practice

- Average/Good or Very good
- Private: 17
- Public: 14
- Below average
- Private: 9
- Public: 9

Two missing data
Better score for the Labs with high volume of Pap cytology/month

- Above average: 11 labs
- Below average: 6 labs
- <800: 6 labs
- 800-3000: 9 labs
- >3000: 6 labs

Legend: Yellow = Below average, Blue = Average/Good or Very good
Literature key issues

• Sporadic maintenance or lack of corrective action for equipements

• Dyes:
  • Absence of daily control of staining quality and hematoxylin filtering
  • Absence of replaced regularly dyes
  • Absence of lot-to-lot evaluation for different lots of reagents

• Absence of communication within the staff

• Staff training differences (continuing education program should be provided)
Laboratory Equipments

- Spreading cell automates
- Staining automates:
  - Dedicated for BD and Hologic
  - Multistainer: Shandon, Gemini Varistain ES, Sakura, ..
- Automated scanning devices and computer-assisted microscopy: Imager™ and FocalPoint™ systems

Better results for staining automates with BD

No participant using automated scanning devices for this EQA

- Instrument maintenance +++

Shift of the spot
Influence of Reagents

- **Fixatives:** methanol, ethanol, spray (if PEG in spray fixative, removing in alcohol prior to rehydration)

- **Dyes:**
  - Hematoxylin
  - Orange G, EA50 anionic stains

- **Alcohol baths**
  - Alcohol baths (ethanol 96%, then 100%) should be changed regularly to achieve a **good differentiation** and **stain transparency**.
  - The last alcohol (100%) and xylene baths should be absolutely clean and water-free. Any remaining water can cause the slide to become decolored

- **Mounting and coverslipping**

```
<table>
<thead>
<tr>
<th>Fixative</th>
<th>BD</th>
<th>Hologic</th>
<th>Mayer</th>
<th>Gill</th>
<th>Harris</th>
</tr>
</thead>
<tbody>
<tr>
<td>Below average</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>Average/Good or Very good</td>
<td>7</td>
<td>4</td>
<td>1</td>
<td>1</td>
<td>23</td>
</tr>
</tbody>
</table>
```

Harris hematoxylin (45%)

“Cornflakes” artifacts
Literature key factors influencing the quality of nuclear staining

Nuclear staining depends upon:
- **Optimal fixation**
- **Type of hematoxylin**
- **Time in hematoxylin**
- **Degree of differentiation and bluing**
- **Rinsing baths**

Progressive method is easier to perform with acid hematoxilin (ideal pH~3), with a slow rate of uptake, no overstaining, no differentiation but requiring bluing.

Hematoxylin should be:
- daily filtered
- renewed when too old
- not diluted (water must be drained from racks prior on immersion in dye)

Regressive method (without acetic acid), is more difficult, with a rapid rate of uptake, possible overstaining and requiring differentiation and bluing:

- If nuclei are too dark, use 0.5% HCL in 70% ethanol
- If nuclei are not enough blue, bluing in acid tap water or in lithium carbonate bath
Staining Result with ThinPrep®

= Filtration technique

• Best colour spectrum
• Endocervical cells, not always present
• Some holes at the surface of the spot, due to mucus on the filter
Staining Results with SurePath®

= Sedimentation technique

• Quality of the dispersion of the cells on all the spot

• Cytoplasm folded at the edges of cells

• Dense orange colour associated with keratinization not seen, probably due to EA50 and Orange G mixture
Staining Results with Novacyt®

= Sedimentation technique

• Majority of the slides has defects:
  – Inadequate dispersion of cells with aggregates and « crescent moon » pictures
  – Cell aggregates poorly fixed
  – Poor details of chromatin, with brownish nuclei.
Key messages (1)

• Best diagnosis begins with best preparation and staining of the slides. Poor quality of staining may lead to a delayed or inappropriate diagnosis.
• EQA controls accuracy of analytical methods.
• This first French EQA Pap test shows that there is room for improvement for some Labs.
Key messages (2)

• BD allows to obtain a more regular staining quality and dispersion of the cells.
• Hologic slides have a more subtle spectrum of cell colour and obtain the best score.
• For genotyping and ICC using Ki67 and P16, only Hologic and BD are recommended for their analytical and stability performances.
Acknowledgments

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