Study of cap pap versus conventional pap in suspicious cervical lesions

Neha Batra1*, Agarwal2, P. M Santwani3

1Assistant Professor, Department of Pathology, Punjab institute of Medical Sciences, Jalandhar.
2Associate professor, 3Professor and head, department of pathology, Shri MP Shah medical college, Jamnagar

INTRODUCTION
Cervical cancer ranks as the most common cancer among women in India, and most frequent cancer among women between 15 and 44 years of age. About 7.9% of women in the general population are estimated to harbor cervical HPV infection at a given time, and 82.5% of invasive cervical cancers are attributed to HPVs16 or 18. Cervical carcinoma is the only cancer which documents the remarkable effects of screening, in early diagnosis and curative therapy on mortality rates. Much credit to this gain belongs to the effectiveness of Pap smear in detecting cervical precancerous lesions and accessibility of the cervix to colposcopy and biopsy. Despite the fact that more than 80% of cervical cancer cases are in developing countries, only 5% of women there have ever been screened for cervical abnormalities. Application of Pap test has resulted in a dramatic reduction of both mortality and morbidity in the countries it was first introduced. The multi-centric study in India evaluated the accuracy of conventional cytology. The sensitivity of the cytology for detection of high-grade lesions was 57%. Despite the high burden of disease and the increasing absolute number of cases due to population growth, there are no organized screening programs for cervical cancer prevention anywhere in India. To improve the detection of precancerous cervical lesions using Pap smear screening, a number of adjunctive tests have been developed including thin layer cytology, use of magnified chemiluminescent screening examination (speculoscopy) combined with Pap smear (Papsure). However, limited by their prohibitive cost factor and unavailability beyond few tertiary care referral centres, these newer technologies have no role in large scale screening programs of developing countries. Some new researchers have proposed a cheap and simple test, viz., cervical acid phosphatase – Pap an icolaou test (CAP-PAP Test; Mark Pap Test) as an adjunct to routine Pap. In this test, abnormal

ABSTRACT
BACKGROUND: CAP Pap test is a new test developed as an adjunct to routine PAP to improve its sensitivity. This test combines a simple biochemical test of enzyme labelling with conventional Pap Test in which abnormal squamous cells of the cervix are labelled for the presence of the Lysosomal enzyme, cervical acid phosphatase (CAP). Objectives: 1. To establish the sensitivity of new test CAP PAP in cervical carcinoma screening. 2. To assess the utility of CAP PAP as an adjunct to routine PAP. MATERIAL AND METHODS: The study comprised of 100 females who were randomly selected. Two smears of each patient were examined by panel of four experts independently and reported. The study was a single centric, random assignment, blinded, 2-group (test and control), and split-sample design to assess safety and efficacy of the new test in comparison with the control for cervical cancer screening in standard Pap test environment. Cervical biopsy was the Gold standard test for the present study. This data was compared and the sensitivity and specificity of the test was noted. RESULT: CAP PAP showed greater sensitivity (100%) as compared to Pap test (75%), while specificity (97.2%) is little lesser than Pap (100%). CONCLUSION: The CAP-PAP Test is a simple diagnostic tool which can be used with routine Pap staining on the same slide adding to the accuracy of routine Pap. This Test could be the future of cervical carcinoma screening.

KEYWORD: Cervical Carcinoma, Screening, sensitivity
Squamous cells of the cervix are labelled for the presence of the Lysosomal enzyme, cervical acid phosphatase (CAP). Demonstration of acid phosphatase in tissues and cells is based on enzyme catalysis of organophosphate substrate, capture of phosphate by a metallic ion (i.e. lead), or an organic radical (aromatic ring) by a diazonium salt, formation of a product which is insoluble at acid pH range (pH<5.0), and precipitation of a colourful, granular deposit at sites of enzyme activity which was available for microscopic examination. The amount is measurable and it is proportional to acid phosphatase activity.

Acid phosphatase is abundant in metabolically active cells in inflammation and malignancy. The alteration of synthesis, processing and trafficking of lysosomal enzymes in malignancy has been demonstrated. A consistent increase of lysosomal enzymes (i.e., prostatic acid phosphatase) has been found in tumor cells in comparison with their normal counterparts. This property contributes to "aggressiveness" of malignant cells. In blood cells, an increase of acid phosphatase activity was found in connection with infection and inflammation (i.e., PMN, monocytes).

In 1986-88, Markovic et al investigated a number of human tissue specimens versus many cytochemical techniques to select candidates for quantitative image analysis. In one of series, cervical smears were exposed to cytochemical techniques for demonstration of lysosomal enzymes. Surprisingly, acid phosphatase activity was found in atypical squamous epithelial cells while normal-looking cells did not present this type of activity. Acid phosphatase activity was inversely proportional to maturity of epithelial cells, and abnormal-looking cells possessed most active acid phosphatase.

The double-staining, single-slide procedure in which acid phosphatase could be stained inside abnormal cervical cells, and counterstained by a modified Papanicolaou (for visualization of classical cytological criteria) was named the Cervical Acid Phosphatase-Papanicolaou (CAP-PAP) Test. Cervical acid phosphatase(CAP) test results in red, granular deposits against a modified Papanicolaou background. CAP is not present in the squamous cells of the normal female genital tract. Endocervical cells and monocytes however, contain CAP (will serve as internal quality control for adequacy of sampling and staining). (Fig 2)

CAP activity increases with the degree of cervical dysplasia .CAP activity is also present in cervical cancer cells, and in HeLa cell line cells derived from human cervical cancer. Control slides made of HeLa cell line cells and buccal cells (COMBO controls) serve as external QC/QA. (Fig 1)

**MATERIAL AND METHODS**

The study comprised of 100 females who were randomly selected according to inclusion and exclusion criteria of study and these were the females whose follow up was available. The study was approved by Institutional ethical committee.

**Inclusion criteria**

1. All women aged 21-70 yrs who have ever had sex & who attended gynaecological O.P.D of tertiary health centre.
2. Females with gynaecological complaints like bleeding/discharge per vaginum were included.
3. All females who attended Pap smear screening camps. (Those without positive history were considered as control)
4. Women who have been attending ART centre.

**Exclusion criteria**

1. Females in menstruation.
2. Those who had a normal Pap smear less than a year ago.
3. Less than 21 years of age.
4. The cervix showing obvious inflammation

The two smears collected from the female The conventional cervical smears were fixed by 100% methanol whereas CAP PAP smear was fixed by fixative made by

- 25 ml Citrate Solution
- 65 ml acetone, and
- 8 ml 37% formaldehyde.

Placed in glass bottle and capped tightly. It
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was stored under refrigeration at 2 to 8 degree Celsius.

**Staining**

First smear was stained as the conventional Pap staining procedure.

Second smear labelled as CAP PAP was stained by following technique.

Staining procedure was same as Pap staining plus steps for staining of enzyme (Cervical Acid Phosphatase). This includes the following steps: marker visualization and counterstaining.(by conventional Pap stain). Slides were fixed at room temp for 50 sec, followed by rinsing in two changes of distilled water. Slides are then air dried and stored in dust free container at 4-6 degree Celsius.

At the time of staining, incubation solution is freshly prepared by mixing

a. 10 drops sodium nitrate
b. 10 drops of Fast Garnet GBC (incubated at room temp for three minutes) in an Erlenmeyer flask containing 46ml pre-warmed distilled water and 2.5ml acetate solution.

c. Ten drops of substrate solution (containing alpha naphthyl ASBI phosphate) was finally added to this mixture. Slides are to be rehydrated and incubated for 45min at 37 degree Celsius in the dark, followed by serial rinsing in running tap water, distilled water, and finally in phosphate buffered saline for 5 min each.

Slides are then counterstained using a modified pap smear with different incubation time in Gill haematoxylin (4 min)
OG-6 (3 min)
EA -65 solutions (5 sec)

Slides were mounted using crystal mount. Eventually evaluated at magnification b/w 100x and 400x.

**Interpretation of CAP PAP smear**

CAP is a brown-red deposit scattered throughout the cytoplasm of "abnormal" cervical cells. PAP staining produces a colour that is combination of original Papanicoloao recommendation: Hematoxylin stains cervical cells nuclei blue, and adds a bluish coloration to the color of cytoplasm.

Orange G (OG-6) stains cervical cell cytoplasm orange (red + yellow). Eosin Alcohol (EA-65) stains cervical cell cytoplasm red. However, Light Green added to the solution of EA, causes green color of cytoplasm.

The "ideal" staining will produce the following results:

CAP -red-brown individual granules scattered through cytoplasm.

Other cytological features (nucleus, nuclei, vacuoles, other granulation) easy distinguishable.

CAP staining produces a red-brown granular precipitate at intracellular sites of enzyme activity. Counterstaining assists presenting cell morphology, cell identification and classification. Two smears of each patient were examined by panel of four experts independently and reported.

**STUDY DESIGN (Statistics)**

The study was a single centric, random assignment, blinded, 2-group (test and control), and split-sample design to assess safety and efficacy of the new test in comparison with the control for cervical cancer screening in standard Pap test environment. Cervical biopsy was the Gold standard test for the present study.

Data was collected as

a. The smears positive by Pap,
b. The smears positives by CAP PAP.

This data was compared and the sensitivity and specificity of the tests was noted. Appropriate statistical tests were applied.

1) Efficacy was measured with primary end points (portion of positive/abnormal specimens detected, and the false negative rate).
2) Accuracy was measured by a. Sensitivity & b. Specificity.

**STUDY DESIGN**

Sample collection (group of cervical cells by Ayres spatula and endocervical brush)

T CAP PAP Split sample PAP

Interpretation of test group Interpretation of control

follow up of positive subjects Close follow positive Subjects

Gold standard diagnostic procedure

Cervical biopsy.

**RESULTS**
One Smear each of 100 randomly selected females were stained by CAP PAP, showed positivity in the form of red granular deposits at the site of enzyme activity inside the cell, while other cellular structures and CAP negative cells were stained by modified Pap method. Endocervical cells and inflammatory cells showed marked cytoplasmic red granular deposits. There were no extra-cellular diffusion but in some cases staining were so intense that it obscured the nuclear details. All inflammatory cells contain acid phosphatase (Lysosomal activity is increased during inflammation) However, inflammatory cells (PMN, monocytes) are smaller than cervical epithelial cells, and possess many other morphological characteristics for easy identification. Metaplastic cells were always positive for the red granular deposit which could be identified in low power, however for nuclear details high power was used. For analysis, smears showing CAP positive squamous cells with nuclear atypia or enlargement were considered positive by CAP PAP. (Fig 4)

**Reporting of cap pap**

The CAP-PAP Test is intended to replace Pap test for primary screening of cervical smears for cervical dysplasia and/or cervical cancer. The test should not be used for exfoliative cytology of vaginal smears. After screening, a technician at primary health centre will have to categorize the smear into one of two options: POSITIVE meaning there are signs of atypia and marker activity is present. NEGATIVE meaning there is no signs of atypia and no marker activity observed. Smears having CAP positive squamous cells with nuclear enlargement or atypia were considered positive by the CAP PAP test. Out the 100 smear examined 30 came out to be positive according to the criteria of positivity (by CAP-PAP) which included 21 reported as positive for intraepithelial malignancy by Pap smear.(second smear stained by conventional Pap). Out of 100 selected smears, 21 smears were reported abnormal on Pap smear while 30 smears showed positivity for CAP PAP. W.e.f table 1 Cervical biopsy which was Gold standard test for the present study, was performed on all the 30 females positive by CAP PAP. Table 2 shows that cervical biopsy confirmed the findings of the CAP PAP smear in 28 smears. While two were found to be negative for pre-malignant lesions. So, these two cases were reported false positive on CAP PAP. The two smears reported falsely positive on CAP PAP were:

- One with intense inflammation where marker positivity in inflammatory cell was so intense, that morphology of squamous epithelial cells was obscured.(Fig 3)
- In second case, many endocervical cells were mis interpreted as malignant squamous cells because of their inherent positivity.

Nine smears which were reported normal or negative for atypical squamous cells on Pap smear showed positivity for CAP PAP (In 8 cases reparative changes were there and one case of acute cervicitis with Metaplastic cells was observed.) In these females follow up cervical biopsies were examined. As per table no 3, nine cases which were negative on Pap showed CAP PAP positivity and various type of abnormality on cervical biopsy. These patients needed long term follow up.

**Statistical Analysis**

To find out the accuracy of Pap and CAP PAP test, the sensitivity and specificity of the tests were calculated. (Table 4 and 5) CAP PAP showed greater sensitivity(100%) as compared to Pap test(75%), while specificity(97.2%) is little lesser than Pap(100%).

**DISCUSSION**

The CAP-PAP Test is a new double-staining, single-slide microscopic method for the diagnosis of cervical dysplasia. In this technique acid phosphatase enzyme is to be labelled in abnormal cervical epithelial cells on Pap smears stained by conventional Papanicolaou technique and, by improving visibility of abnormal cells to improve human
perception of abnormal cells and interpretation of Pap smears.
Increasing accuracy (better sensitivity and at least equivalent specificity with Pap test) the CAP-PAP test could reduce false negative readings of the conventional Pap test.
Normal cervical epithelium contains acid phosphatase, but the enzyme activity is gradually reduced subsequently to the maturation from basal to intermediate cells. Superficial cells are always negative. However, abnormal intraepithelial growth such as hyperplasia, dysplasia (mild and severe) and cancer are always positive. This discrepancy between enzyme activity inside normal and abnormal cells, makes cervical acid phosphatase a natural biomarker for detecting abnormal growth. CAP activity is absent in all normal squamous cells excoriated from superficial, intermediate of outer basal layer of cervical epithelium In the same time, CAP activity was present in all squamous cells showing morphological signs of cervical cell abnormality. Markovik et al (2003) demonstrated that CAP PAP resulted in Doubling the portion of positive/normal slides referred to pathologist (27%: 13%) Significant reduction in the portion of false negative slides found at rescreen (5%: 9%) Increase of the portion of disease positive slides (true positive) identified by pathologists (17%: 8.2%)
Deb et al (2008) demonstrated a sensitivity of 100% and specificity of 89%, as compared to the conventional Pap stain. The predictive value of a positive CAP-PAP test was 50%, while it was 100% for a negative test. Overall, there were no false negative in the study, while false positive were 11%.
Findings of present study correlate with other studies, in that CAP PAP test is a better screening test as compared to Pap because of increase in sensitivity reported. Cases missed on Pap smear but positive on CAP PAP were 9 which included seven reported as reparative change with inflammation who were advised repeat pap smear.
These Patients with inflammatory Pap smears can transform into cervical dysplastic lesion because of repeated HPV infection and hence these patients will need further evaluation. The CAP PAP will be able to detect these dysplastic changes at early stages. In equivocal morphology (ASCUS), when between Pap(+) and Pap(-) result cannot be decided ,a repetition of the test was advised within next three months following intensive anti-inflammatory therapy. The CAP-PAP test could provide valuable information of the effect of therapy on reduction of inflammatory cells, and repairation of cervical epithelium (reduction of acid phosphatase activity).
However, the test has some limitations of false positivity because of positivity in endocervical cells and intense staining obscuring nuclear details. CAP PAP cannot be reported without evaluating nuclear feature on pap smear.
CONCLUSION
Table: 1 Comparing cap pap and pap findings (n= 100)

<table>
<thead>
<tr>
<th>Lesions</th>
<th>Pap</th>
<th>Cap pap</th>
<th>Positive</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>27</td>
<td>27</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acute cervicits</td>
<td>42</td>
<td>1</td>
<td>41</td>
<td></td>
</tr>
<tr>
<td>Reparative changes</td>
<td>10</td>
<td>8</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Ascus</td>
<td>3</td>
<td>3*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lsi</td>
<td>9</td>
<td>9*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hsi</td>
<td>6</td>
<td>6*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
<td>3</td>
<td>3*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>100(21*)</td>
<td>30</td>
<td>70</td>
<td></td>
</tr>
</tbody>
</table>

Table: 2 Showing comparison of pap, cap pap and cervical biopsy in all cap positive smears (n=30)

<table>
<thead>
<tr>
<th>Pap</th>
<th>Cap pap(positive)</th>
<th>Cervical biopsy Negative for malignancy</th>
<th>Positive for malignancy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Cin i  Cin ii Ciniii Scc</td>
<td></td>
</tr>
<tr>
<td>Acute cervicits(01)</td>
<td>Positive (01)</td>
<td>01</td>
<td>-</td>
</tr>
<tr>
<td>Reparative changes(10)</td>
<td>Positive(08)</td>
<td>01</td>
<td>06  01</td>
</tr>
<tr>
<td>Ascus(03)</td>
<td>Positive(03)</td>
<td>-</td>
<td>03</td>
</tr>
<tr>
<td>Lsi(09)</td>
<td>Positive(09)</td>
<td>-</td>
<td>- 07 02</td>
</tr>
<tr>
<td>Hsi(06)</td>
<td>Positive(06)</td>
<td>-</td>
<td>- 06</td>
</tr>
<tr>
<td>Squamous cell carcinoma(03)</td>
<td>Positive(03)</td>
<td>-</td>
<td>- - 03</td>
</tr>
<tr>
<td>Total</td>
<td>30</td>
<td>02</td>
<td>28</td>
</tr>
</tbody>
</table>
As indicated by the sensitivity of 100%, CAP PAP fulfills the criteria of screening test, so this test could be very useful as an quick, cheap and efficient method of large scale screening. CAP positivity helps in easy and early detection of abnormal cells because of red coloured granules which are easily identified and thus speeds up the screening process.

The test promises a great future at primary level health centres in India where trained persons are not available, technicians can be easily trained for identification of abnormal smears.

**Table: 3 showing comparison of conventional pap, cap pap and cervical biopsy. (N=9)**

<table>
<thead>
<tr>
<th></th>
<th>PAP negative</th>
<th>CAP PAP</th>
<th>Cervical Biopsy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute cervicitis with metaplastic cells (01)</td>
<td></td>
<td>Positive (01)</td>
<td>Negative for malignancy (01)</td>
</tr>
<tr>
<td>Reparative changes (08)</td>
<td>Positive (08)</td>
<td>CIN I (06)</td>
<td>CIN II (01)</td>
</tr>
<tr>
<td>TOTAL</td>
<td>09</td>
<td>09</td>
<td></td>
</tr>
</tbody>
</table>

**Table: 4 Statistics of pap smear group (n=100)**

<table>
<thead>
<tr>
<th>Disease present</th>
<th>Disease absent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test positive</td>
<td>21 (a)</td>
</tr>
<tr>
<td>Test negative</td>
<td>7 (c)</td>
</tr>
</tbody>
</table>

Sensitivity = \( \frac{a}{a+c} = \frac{21}{28} = 75\% \)

Specificity = \( \frac{d}{b+d} = \frac{72}{72} = 100\% \)

**Table: 5 Statistics of cap test (n=100)**

<table>
<thead>
<tr>
<th>Disease present</th>
<th>Disease absent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test positive</td>
<td>28 (a)</td>
</tr>
<tr>
<td>Test negative</td>
<td>0 (c)</td>
</tr>
</tbody>
</table>

Sensitivity = \( \frac{a}{a+c} = \frac{28}{28} = 100\% \)

Specificity = \( \frac{d}{b+d} = \frac{70}{72} = 97.2\% \)

**Fig: 1 Mark Pap Combo control slides, several marker positive Hela cervical carcinoma cell line and one negative normal buccal epithelial cells. CAP PAP (Oil immersion).**

**Fig: 2 Endocervical cells showing CAP positivity which acts as an internal control. (CAP PAP, Oil immersion)**

**Fig: 3 Acute cervicitis. Smear showing many neutrophils and one metaplastic cell showing CAP positivity. (CAP PAP, Oil immersion)**

**Fig: 4 Smear showing large cluster of malignant squamous epithelial cells, showing high degree of CAP positivity. (CAP PAP, Oil immersion).**

**REFERENCES**

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