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ABSTRACT

Aims: Despite many improvements, cervical cancer screening is still subject to shortcomings. Diagnostic accuracy may improve by using molecular biological techniques, requiring RNA of superior quality. This study determined the effect of SurePath fixation on RNA integrity to assess the suitability of clinical samples collected in this medium for RNA-based molecular assays.

Methods: RNA isolation was performed on fresh and fixed HeLa cells and exfoliated cervical cells fixed in SurePath. The RNA integrity was evaluated by analysis of ribosomal RNA as an indicator of quality. The effect of SurePath preservation on PCR amplification was evaluated by real-time reverse transcriptase (RT)-PCR.

Results: In contrast to unfixed cells, SurePath-fixed cells yielded less and severely degraded RNA, as shown by the absence of ribosomal RNA bands. RNA derived from SurePath-fixed cells showed poor real-time RT-PCR amplification characteristics, as evidenced by the absence correlation between threshold values and log cDNA concentration.

Conclusions: Implementation of molecular biology in a clinical context is on the rise and may alleviate shortcomings in current screening and diagnostics. This study shows that SurePath fixation gives rise to highly fragmented RNA with insufficient quality for further reliable analysis by standard real-time RT-PCR applications. The increasing prominence of molecular screening stresses the importance of this finding, which must be considered in relation to choice of an appropriate liquid-based cytology system.

Cervical carcinoma, caused by a persistent infection with oncogenic human papillomavirus (HPV) types, is the third most frequent cancer among women worldwide. Cytological screening for well-defined precursor lesions has proven its efficiency, and reduced incidence and mortality significantly. However, the conventional Papancolau test shows limited sensitivity and specificity, and is susceptible to intra- and inter-individual variability. Introduction of new methods such as liquid-based cytology (LBC), and HPV DNA testing and typing, has been important in improving screening. Furthermore, stored LBC samples provide specimens for ancillary testing.

Increased knowledge concerning the molecular biology of cervical carcinogenesis raises expectations that biomarkers will result in more accurate diagnosis of cervical cancer. Moreover, the emergence of molecular medicine has resulted in the increased use of RNA in clinical diagnostics. Using RNA transcripts of known genes involved in carcinogenesis as biomarkers requires the ability to obtain high-quality RNA, representative of the disease from routine clinical samples.

Worldwide, two methods are commonly used in liquid-based cervical cytology. The SurePath method (Tripath Imaging, Burlington, North Carolina, USA) uses an ethanol-based preservative for the fixation of clinical specimens, and is currently the most frequently used LBC collection medium in Belgium. The Thinprep technique (Cytyc, Marlborough, Massachusetts, USA) applies the methanol-based fixative PreservCyt. Residual material from PreservCyt samples is suitable for DNA and RNA isolation and subsequent analysis by PCR and real-time reverse transcriptase (RT)-PCR. Although cells fixed in SurePath render DNA suitable for routine HPV detection and typing, it is less stable than when obtained from cells fixed in PreservCyt. Moreover, reduced RNA recovery and RNA degradation in CaSki cells fixed in SurePath have been reported.

The present study was designed to determine the effect of SurePath fixation on RNA integrity to evaluate the suitability of clinical samples collected in this medium for RNA-based molecular assays.

RNA recovery and integrity from SurePath-fixed HeLa cells was evaluated. Subsequently, the RNA conditions from routinely taken clinical samples, collected in SurePath medium, were assessed.

METHODS

Cell culture

The human cervical cell line HeLa (ATCC CCL-2), which contains 10–50 copies of integrated HPV-18, was grown under a humidified carbon dioxide atmosphere in Dulbecco’s Modified Eagle’s Medium supplemented with 10% fetal bovine serum, 200 mM l-glutamine, 50 mg/l penicillin/streptomycin, 50 mg/l gentamicin, non-essential amino acids (Gibco, Carlsbad, California, USA) and 50 mg/l amphotericin (Sigma-Aldrich, Milan, Italy).

Cultures were harvested at 90% confluence and resuspended in FBS, acting as fresh, unfixed control cells for immediate RNA extraction, or in 8 ml SurePath collection medium, providing SurePath-fixed cells stored at 4°C for 1 week. Each HeLa control was provided in triplicate.

Clinical samples

Twenty SurePath-fixed clinical samples were obtained from the Laboratory for Clinical Pathology (Labo Lokeren, campus RIATOL, Antwerp, Belgium). The study was performed in
Table 1  Primer sequences used in the real-time RT-PCR assay

<table>
<thead>
<tr>
<th>Target</th>
<th>Sequence</th>
<th>Amplicon length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPRT</td>
<td>5'-GGCGATATATCAGTTTCAAGAAATGTTT-3'</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>5'-AGTGGCTTATATCTTCAACCATCCTGT-3'</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>5'-CCACATGGCTGACGACACCAT-3'</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td>5'-GTGACAGGGGCCCACAT-3'</td>
<td></td>
</tr>
</tbody>
</table>

HPRT, hypoxanthine phosphoribosyltransferase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; F, forward; R, reverse.

accordance with the guidelines of the local ethical committee. Informed consent was not obtained because no risks were attributable to the investigation. Study-specific patient identification codes were used to ensure patient confidentiality.

Extraction methods
RNA was extracted from 2 ml of the total sample. Two total RNA extraction procedures were tested for their ability to provide RNA suitable for real-time RT-PCR analysis; the reagent-based method TRIzol (Invitrogen, Carlsbad, California, USA) and the column-based technique SV Total RNA Isolation System (Promega, Madison, Wisconsin, USA) according to the manufacturer’s instructions. RNA was resuspended in RNase-free water and stored at −20°C.

RNA analysis
RNA concentration and purity were monitored by UV spectrophotometry. The RNA integrity was evaluated by the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, California, USA) using the RNA 6000 Nano Lab Chip (Capiler Life Sciences, Hopkinton, Massachusetts, USA) in order to assess the quality of the extracts for downstream applications. The technique is based on analysis of evident 5S, 18S and 28S ribosomal RNA. A 1 µl volume of each RNA extract was analysed according to the manufacturer, and electropherogram profiles were generated.

Reverse transcription
Prior to cDNA synthesis, DNA was removed from the total RNA extracts. An amount of 12 µl DNase-treated total RNA was reversed transcribed in a 20 µl reaction volume using the Transcriptor First Strand cDNA synthesis kit (Roche Applied Science, Indianapolis, Indiana, USA) according to the manufacturer’s instructions. A control for elimination of contaminating genomic DNA was provided by DNase-treated RNA samples that were handled in the same manner, with omission of RT enzyme (‘no RT-control’).

Real-time RT-PCR
Real-time RT-PCR analyses were performed using the LightCycler system based on SYBR Green I methodology (Roche Applied Science). For the generation of standard curves, serial dilution series of cDNA were obtained from fresh and SurePath-fixed HeLa cells and exfoliated cervical cells. PCR efficiencies were tested for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (NM_002046) and hypoxanthine phosphoribosyltransferase (HPRT) (M31642). Specific primers for these housekeeping genes were designed using Primer Express 2.0 software (Applied Biosystems, Foster City, California, USA) (table1).

For the LightCycler reaction a mastermix of the following reaction components was prepared to the indicated end-volume (20 µl): 9 µl PCR-H2O, 1 µl forward primer (0.5 µM), 1 µl reverse primer (0.5 µM), 4 µl LightCycler Mastermix (LightCycler Fast Start DNA MasterPLUS SYBR Green I kit; Roche Applied Science) was filled in the LightCycler glass capillaries and 5 µl cDNA was added as the PCR template. The following protocol was used: denaturation programme (95°C for 10 min), amplification and quantification programme repeated 50 cycles (95°C for 10 s, 58°C for 10 s, 72°C for 10 s, with a single fluorescence measurement), melting curve programme (55–95°C, with a heating step of 0.1°C/s and a continuous fluorescence measurement), and finally a cooling step to 40°C. Controls in the real-time RT-PCR reaction included RNA without RT (control for DNA carry over), and omission of template (control for PCR contamination). All experiments were done in triplicate and crossing point (CP) values obtained were plotted against log cDNA concentration. CP is defined as the cycle at which the fluorescent signal has risen significantly above the background.

Statistical analysis
Data were analysed using SPSS 14.0 for Windows 2000 (SPSS, Chicago, Illinois, USA). Independent-samples t test was used to compare RNA concentration and purity for the two RNA extraction methods and to assess the effect of fixation on these parameters, regardless of the isolation method.

RESULTS
RNA extraction
Mean concentrations and purities of RNA extracted by the two total RNA isolation techniques are given in table 2.

Table 3 compares the influence of fixation on the RNA concentration and purity, and compares both RNA extraction methods.

Total RNA preservation
To determine the effect of fixation on RNA integrity, RNA extracted from fresh cells was compared with RNA extracted after SurePath fixation by analysis of 5S, 18S and 28S ribosomal RNA. When total RNA from fresh, unfixed HeLa cells was analysed (fig 1A), peaks representing the rRNA bands were clearly identifiable with a typical 28S/18S rRNA ratio higher than 1. These features indicate intact, non-degraded RNA.

The electropherogram obtained from total RNA extracted from SurePath-fixed cells showed severe degradation, indicated by the absence of ribosomal RNA bands and a clear left-sided shift to the area representing short and fragmented RNA (fig 1C–H).

RNA extraction methodology did not affect RNA integrity as indicated by assessment of the ribosomal RNA profiles.
Table 3  Comparison of the influence of fixation on the RNA concentration and purity regardless the isolation method, and comparison of the two different RNA extraction methods

<table>
<thead>
<tr>
<th></th>
<th>Unfixed samples</th>
<th>Fixed samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean RNA yield (ng/µl)*</td>
<td>216.13 (226.73)†</td>
<td>36.58 (38.60)‡</td>
</tr>
<tr>
<td>Mean A₂₆₀/A₂₈₀ ratio</td>
<td>2.02 (0.15)§</td>
<td>1.69 (0.13)¶</td>
</tr>
<tr>
<td>TRIzol</td>
<td>164.22 (166.38)§</td>
<td>6.87 (5.99)§</td>
</tr>
<tr>
<td>SV Total</td>
<td>508.23–28.72</td>
<td>2.12–18.89</td>
</tr>
<tr>
<td>Mean A₂₆₀/A₂₈₀ ratio</td>
<td>1.85 (0.23)¶</td>
<td>1.72 (0.15)¶</td>
</tr>
</tbody>
</table>

Values in parentheses are SD.
*RNA concentration was calculated from the absorbance at 260 nm.
†The difference in concentration between control and fixed cells is statistically significant (p = 0.005).
‡The difference in A₂₆₀/A₂₈₀ ratio between control and fixed cells is statistically significant (p = 0.001).
§The difference in yield between the two extraction methods is statistically significant (p = 0.008).
¶The difference in A₂₆₀/A₂₈₀ ratio between TRIzol and SV Total RNA Isolation System is not statistically significant (p = 0.132).

Real-time RT-PCR

Amplifiable cDNA was detected by real-time RT-PCR with primers for HPRT and GAPDH. TRIzol extraction rendered a significantly higher RNA yield (p<0.0001) than the SV Total RNA Isolation System; TRIzol extracts were used for real-time RT-PCR. The housekeeping gene assays showed reproducible differences concerning the amplifiable character of cDNA from RNA from cells SurePath-fixed compared with RNA from the unfixed control samples. In case of fresh unfixed HeLa cells, a strong linear correlation between the number of crossing points and the log of the input cDNA amount was shown (fig 2A,C). When real-time RT-PCR analysis was performed for SurePath-derived RNA, the profile obtained did not show a significant correlation between the CP values and the log of the cDNA concentration (fig 2B,D). Table 4 shows the R² values and standard curve slopes for unfixed and fixed samples.

Discussion

Well-conducted conventional screening for cervical carcinoma, based on cytology, has proven its efficiency, despite many shortcomings related to sensitivity and specificity.1 Introduction of LBC has been important in improving screening and is used to a great extent in clinical patient management.8–10 Moreover, it provides specimen for ancillary molecular biological testing.11 Increased knowledge concerning the molecular biology of cervical cancer has led to new methods and optimised diagnostic accuracy.7 19

Nowadays, RNA-based techniques are gaining importance in clinical diagnostics. An important aspect in cervical carcinogenesis is the expression of viral oncoproteins, which are necessary for progression to malignancy and maintenance of the cancerous phenotype.20 Therefore, detection of RNA transcripts of genes involved in oncogenesis may enhance the ability to differentiate between asymptomatic HPV infections and infections associated with high-risk lesions and cervical carcinoma, serving as a better risk evaluation factor than mere DNA detection.10 11 16 20–25

Growing interest in using RNA transcripts as biomarkers of disease, and the increased use of real-time RT-PCR for clinical purposes, implies that effects of fixatives on the RNA integrity and utility of the preserved RNA should be carefully assessed.10 13 This study determined the effect of SurePath fixation on the RNA integrity, in order to evaluate the suitability of clinical samples collected in this medium for use in RNA-based molecular biological assays.

Spectrophotometric analysis showed that SurePath fixation significantly reduces RNA yield (table 3). Since TRIzol reagent constantly rendered significantly higher RNA concentration (p<0.0001) than the column-based method, TRIzol extracts were used for real-time RT-PCR. RNA integrity analysis showed that the profiles of RNA from SurePath-fixed cells (fig 1C, D–H) were notably different from those obtained from the unfixed control sample (fig 1A). Extensive RNA degradation in the case of SurePath storage led to loss of amplifiable templates in the RNA population, paralleled by loss of amplicons and an increase in threshold values (fig 2A–D). This was confirmed by the significant difference in R² (p<0.0001) and standard curve slopes (p<0.0001) between unfixed and fixed cells, indicating an absence of linearity between CP and log cDNA concentration for fixed material (table 4). In general, the efficiency of a real-time RT-PCR reaction decreases with increasing amplicon size; therefore, the amplicon length in this study was a constraint, providing maximal PCR efficiency (table 1).22 This study does not comprise an extensive longitudinal assessment of RNA conditions to evaluate its candidacy for real-time RT-PCR analysis after various fixation periods. However, our experiments (data not shown) and others,19 performing integrity analyses throughout a time frame (0.1–504 h), indicate that shortening of storage time does not preserve the RNA quality. In clinical laboratorones, LBC samples are primarily processed for cytological purposes and leftovers can be directed to ancillary molecular testing no sooner than a few days after sampling. As SurePath fixation immediately induces RNA fragmentation, potential implications for RNA-based molecular techniques in a LBC system using SurePath seem evident.

The exact compositions of the LBC collection media are not in the public domain and therefore it is difficult to speculate about the biochemical basis of this extensive RNA degradation. SurePath contains less than 24% ethanol, whilst PreservCyt contains 30–60% methanol. It may be the concentration of ethanol in SurePath that is insufficient for prevention of RNA degradation, whilst sufficient for preservation of the cellular architecture. Although the alcoholic reagent is known to be an excellent fixative for the preservation of nucleic acids, experiments evaluating nucleic acid recovery following storage in high concentrations of ethanol (95%) also failed to yield RNA.15

Table 4  Correlation coefficients and standard curve slopes calculated for each housekeeping gene by real-time RT-PCR for the unfixed and fixed samples

<table>
<thead>
<tr>
<th>Target</th>
<th>Sample</th>
<th>R²</th>
<th>Slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>Unfixed</td>
<td>0.98 (0.02)†</td>
<td>−2.67 (0.21)†</td>
</tr>
<tr>
<td></td>
<td>Fixed</td>
<td>0.22 (0.17)‡</td>
<td>0.49 (0.32)‡</td>
</tr>
<tr>
<td>HPRT</td>
<td>Unfixed</td>
<td>0.99 (0.01)‡</td>
<td>−3.37 (0.13)‡</td>
</tr>
<tr>
<td></td>
<td>Fixed</td>
<td>0.23 (0.14)‡</td>
<td>−0.09 (0.69)‡</td>
</tr>
</tbody>
</table>

Values are means (SD). The correlation coefficient R² calculated by the instrument is an indication of the quality of the fit of the standard curve to the standard data plotted. Values closer to 1 indicate a better fit of the data to the line. The slope of the standard curve is related to the average efficiency of amplification and may be used to calculate PCR efficiency.

†The difference in the R² parameter between unfixed and fixed samples, for both housekeeping genes, is statistically significant (p<0.0001).
‡The difference in standard curve slope between unfixed and fixed cells, for GAPDH and HPRT, is statistically significant (p<0.0001).

Original article

Implementation of advanced molecular biology in a clinical context is on the rise and may alleviate shortcomings in current screening and diagnostics. RNA as a potential target for routine clinical diagnostics may improve sensitivity, reproducibility and specificity compared with DNA. Cervical cells used in cytology might be an important source of molecular information. Therefore, it could be favourable that fixatives used for collection and processing of cytological specimens maintain RNA quality. This study shows that SurePath fixation gives rise to highly fragmented RNA with an insufficient quality for further analysis by real-time RT-PCR.

**Acknowledgements:** Dr Eugene Bosmans is acknowledged for providing HeLa cells.

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**Competing interests:** None.
REFERENCES


Figure 2  Real-time RT-PCR analysis. Standard curves for HPRT and GAPDH using cDNA obtained from cDNA from fresh and SurePath-fixed HeLa cells. The R² correlation coefficient, calculated by the instrument, is an indication of the quality of the fit of the standard curve to the standard data points plotted. Values closer to 1 indicate a better fit of the data to the line. The slope of the standard curve is directly related to the efficiency of amplification and can be used to calculate the PCR efficiency (E). (A) A strong linear correlation between the crossing point (CP) values and the log of the input cDNA amount (R² correlation coefficient, 1.00; E, 95%) was obtained for HPRT using fresh unfixed cells. (B) When using a serial dilution series of HeLa cDNA from SurePath-fixed cells to generate a standard curve for HPRT there was no significant relation between the CP threshold values and the log cDNA concentration (R² correlation coefficient, 0.11; E, 2%). (C) A strong linear correlation between the CP values and the log cDNA input (R² correlation coefficient, 0.98; E, 139%) was obtained for GAPDH using fresh unfixed cells. (D) When using a serial dilution series of HeLa cDNA from SurePath-fixed cells to generate a standard curve for GAPDH, there was no significant relation between the CP threshold values and the log cDNA concentration (R² correlation coefficient, 0.16; E, 0.58%).

Take-home messages

- SurePath fixation of cervical cells gives rise highly fragmented RNA with an insufficient quality for analysis by real-time RT-PCR.
- Implementation of molecular biology in a clinical context is on the rise and may alleviate shortcomings in current screening and diagnostics.
- SurePath-dependent RNA degradation must be considered in relation to choice of an appropriate liquid-based cytology system.

REFERENCES

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CD56 and TIA-1, but not CD4, CD5, CD7, CD8, CD10, CD20, CD30 or granzyme B. The proliferation fraction as determined by Ki-67 is at 70%. Surprisingly, staining for cytokeratin cocktail AE1/AE3 highlighted many foci of LEL formed by the infiltration of the residual prostatic acini by lymphoma cells. The tumour cells were positive for EBV-encoded mRNA (EBER) by in situ hybridisation, while PCR study for T-cell receptor gamma chain gene rearrangements revealed polyclonal results.

Eight days after the transurethral resection of the prostate, nasal biopsy revealed heavy infiltration by small to medium-sized atypical lymphocytes in diffuse pattern without angioinvasion or necrosis. Immunohistochemically, these atypical lymphocytes expressed CD3 and CD56 but not CD20, the same histopathology and immunophenotype as that of a prostatic lesion except for the absence of LEL in the nasal tumour either by histopathology or cytokeratin immunostaining. Bone marrow biopsy confirmed marrow involvement. The patient was refractory to two courses of polychemotherapy and died with progressive disease 2.8 months after diagnosis.

LELs very likely represent the neoplastic equivalent of the association of B-cells with dome epithelium that characterises MALT and the result of specific “tropism” of the CCL cells for epithelium. Ultrastructural studies revealed a tight association of CCL cells and epithelial cells leading to structural distortion and disruption of the epithelial cells and their ultimate death. LELs are usually easily found in gastric MALT lymphomas where their presence in a lympho-proliferative lesion is highly suggestive, but not, on its own, diagnostic of lymphoma. Lymphoma of the prostate is very uncommon and most cases in the literature represent secondary involvement after complete staging as in our case. Primary prostatic MALT lymphoma is extremely rare and cases with formation of LELs in prostatic glands have been described. In enteropathy-associated T-cell lymphoma, infiltration of the epithelium of individual crypts is present in most cases without formation of LEL. The intestinal mucosa adjacent to the tumours usually shows features of enteropathy including increased intra-epithelial lymphocytes but not LEL. Our case is unique in that it is the first case of NK-cell or T-cell lymphoma of the prostate, nasal secretary glands. Lymphoma cells formed LELs around prostatic acini but not in nasal secretory glands.

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Competing interests: None declared.
Patient consent: Informed consent was obtained for the publication of the details in this report.
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REFERENCES

There was an error in the title and nomenclature of a paper published in the July 2007 issue of the journal (Zheng H, Murai Y, Hong M, et al. Jamestown Canyon virus detection in human tissue specimens. J Clin Pathol 2007;60:787–93.) The correct title should be as follows: JC virus detection in human tissue specimens. The journal thanks Dr James C Clayton for pointing out the error. A corrected PDF is available at http://jcp.bmj.com/supplemental
doi:10.1136/jcp.2007.047266.cor1

There was an error in the author order of an article published in the January issue of the journal (Effects of fixation on RNA integrity in a liquid-based cytology setting. J Clin Pathol 2008;61:132–7.) The correct order is as follows: CAJ Horvath, G Boulet, S Sahebali, C Depuydt, T Vermeulen, D Vandend Broeck, A Vereecken, J Bogers.
doi:10.1136/jcp.2006.040915.cor1