Ethanol fixation of lymphoma samples as an alternative approach for preservation of the nucleic acids*

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Molecular methods play an important role in diagnostic pathology of lymphomas. PCR based demonstration of clonality or detection of a specific chromosomal translocation may determine the exact classification of the lymphoma. Hence the final diagnosis may depend on the quality of preserved nucleic acids in the bioptic specimen. The integrity of DNA and RNA may be damaged by formalin fixation, which destroys the nucleic acids by fragmentation. Therefore, a portion of each lymphoma sample should be frozen.

To substitute freezing techniques we utilized ethanol as a fixative, which preserves nucleic acids. We compared PCR and RT-PCR products from lymphoma samples, which were differently pre-treated by ethanol fixation, formalin fixation and freezing.

The ethanol fixed samples retained a high quality of both DNA and RNA and provided reproducible PCR products similar to frozen samples and significantly better then those extracted from formalin fixed samples.

We may recommend ethanol as a complementary fixative for all pathology laboratories where deep freezing in not routinely available.

Key words: Ethanol, fixation, DNA, lymphoma.

In pathology practice, there are several procedures how to protect samples against autolytic changes. Formalin is the most convenient fixative for routine surgical pathology practice. It optimally preserves the tissue structure for morphologic investigations. Proteins fixed by formalin are usually suitable for immunohistochemical investigation. However, there is a growing need of molecular methods in diagnostics, particularly PCR and RT-PCR. For this purpose formalin fixation is not optimal due to formalin deleterious effect on the integrity of nucleic acids (NA). It damages NA by fragmentation depending on formalin acidity and on time of exposure [8].

Formalin may degrade nucleic acid to such an extent that the sample becomes useless for diagnostic PCR analysis. In our laboratory we encountered such an undesirable phenomenon many times, especially in the samples fixed in non-buffered formalin or in samples fixed over weekends (received on Fridays, about 20% of all samples). Thus, negative consequences of formalin fixation have a deleterious impact in such fields of pathology, in which the molecular approach may influence the final diagnosis.

As an example, we emphasize the diagnostics of lymphomas, which frequently require PCR investigation of clonality (rearragements of immunoglobulin or T-cell receptor (TCR) genes), and, in some instances a demonstration of lymphoma-specific translocations. Therefore, crucial diagnostic problems may arise, when samples are not appropriately preserved. Optimally, a portion of any lymphoma sample should be frozen. In spite of that we have to solve many cases (especially in consultation biopsies mailed from other hospitals), in which the whole sample is put into formalin. Allowing such inadequate pre-treatment pathologist may even play a negative role in the final diagnostic process. This problem may be caused by a high cost of freezers of

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adequate size and quality (–80 °C), accessories (CO_2 bombs, supply of liquid nitrogen etc.), and unfortunately, by a lack of interest to improve outputs of a routine pathology diagnostics.

Exploiting the generally known fact, that ethanol protects nucleic acids, we analyzed a series of ethanol fixed samples for DNA and RNA diagnostics. We compared PCR and RT-PCR products from lymphoma samples, which were differently pre-treated by ethanol fixation, formalin fixation and freezing. Our aim was to offer an alternative approach, which saves NA and may be easily performed in any diagnostic laboratory.

Material and methods

Samples. We investigated a group of ten B-cell lymphomas (3 cases of small lymphocytic lymphoma, 2 cases of follicular lymphoma, 2 cases of diffuse large B-cell lymphoma, one by one case of mantle cell lymphoma, marginal zone lymphoma and hairy cell leukemia). Fresh tissue samples were divided to several pieces and processed in the following way:

1. The largest, representative piece of each sample was routinely fixed in 10% buffered formalin for 24 hours and then embedded in paraffin blocks. Beside the NA isolation these samples were used for the morphological diagnosis.
2. One piece of each sample was frozen in liquid nitrogen and stored in –80 °C.
3. One piece was fixed in 75% ethanol for 24 hours and then embedded in paraffin in a similar way as was the case with samples fixed in formalin.
4. In four cases we pre-treated one fragment by "formalin-ethanol" fixation: These samples were fixed for 1 hour in non-buffered formalin, then transferred into ethanol and further processed with other ethanol fixed samples. This extra-step was made with the aim to support the conception that formalin degradation of NA is time dependent and may be stopped by changing the fixative for ethanol. The procedure was performed to simulate a situation in which the whole sample is put into formalin but subsequently rescued by ethanol fixation.

DNA and RNA extraction from frozen sections and RNA extraction from paraffin blocks. NA were extracted from frozen or paraffin sections (10 sections per 5 μm) using Tri-Reagent (Life-Technologies, Gibco BRL). Sections from paraffin blocks were previously deparaffinized in xylen and washed in ethanol.

DNA extraction [9]. Sections from paraffin blocks (10 sections per 5 μm) were deparaffinized with xylen three times and washed twice in cool absolute ethanol, then they were digested by 0.1 mg/ml proteinase K in PCR buffer without $MgCl_2$ (Boehringer Mannheim, BRD) at 37 °C overnight. RNase (Boehringer Mannheim) was then added to the supernatant (37 °C for 45 minutes) and NaCl was dissolved in the samples (5 M). DNA was then precipitated by absolute ethanol (–20 °C for 20 minutes) and the DNA pellet was finally washed by 75% ethanol.

Table 1. List of performed PCRs, primers and amplification conditions

Beta-globin /N-myc (two PCRs in one tube)	
Size of products:	Beta-globin: 268 bp N-myc: 428 bp
Primers:	Beta-globin: 5'-GAA-GAG-CCA-AGG-ACA-GGT-AC-3' 5'-CAA-CTT-CAT-CCA-CGT-TCA-CC-3' N-myc: 5'-GAT-GAA-GAT-GAT-GAA-GAG-GAA-3' 5'-TGG-TCC-CTG-AGC-GTG-AGA-AA-3'
Conditions:	Initial denaturation (94 °C, 5 min) 29 cycles of denaturation (94 °C, 1:30 min), annealing (55 °C, 2 min), extension (72 °C, 3 min). Final extension (72 °C, 10 min)
Beta-actin (RT-PCR)	
Size of products:	95 bp
Primers:	5'-GCC-AAC-CGC-GAG-AAG-ATG-AC-3' 5'-GAG-GCG-TAC-AGG-GAT-AGC-AC-3'
Conditions:	Initial denaturation (94 °C, 5 min) 30 cycles of denaturation (94 °C, 1 min), annealing (57 °C, 1 min), extension (72 °C, 1 min). Final extension (72 °C, 10 min)
ABL (RT-PCR)	
Size of products:	209
Primers:	5'-TTC-AGC-GGC-CAG-TAG-CAT-CTG-ACT-T-3' 5'-TGT-GAT-TAT-AGC-CTA-AGA-CCC-GGA-GCT-TTT-3'
Conditions:	Initial denaturation (94 °C, 5 min) 35 cycles of denaturation (94 °C, 1 min), annealing (57 °C, 1 min), extension (72 °C, 1 min). Final extension (72 °C, 10 min)

PCR and RT-PCR. Double PCR was performed on DNA samples with two products: beta-globin (268 bp) and N-myc (428 bp) [3, 4] (Tab. 1).

The degree of DNA fragmentation was estimated by comparison of the PCR products. There were three possibilities (Fig. 1):

1. Presence of both products (beta-globin and N-myc, classified as +/+) signalized, that DNA consists of fragments longer then 428 base pairs.
2. Presence of beta-globin product and absence of N-myc product (classified as +/-) corresponded with DNA degradation to fragments smaller than 428 base pairs but longer than 268 base pairs.

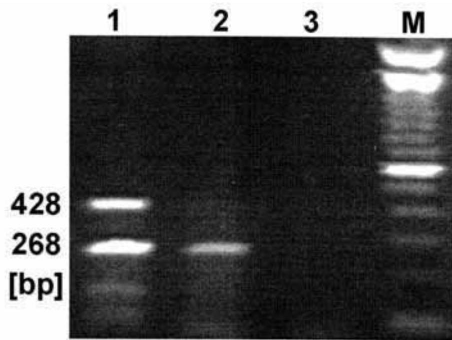

Figure 1. Analysis of the DNA integrity: PCR products of of beta-globin (268 bp) and N-myc (428 bp). Lane 1 (+/+): presence of both PCR products. Lane 2 (+/-): presence of beta-globin, absence of N-myc. Lane 3 (-/-): absence of both PCR products. M – marker (100 bp DNA ladder).

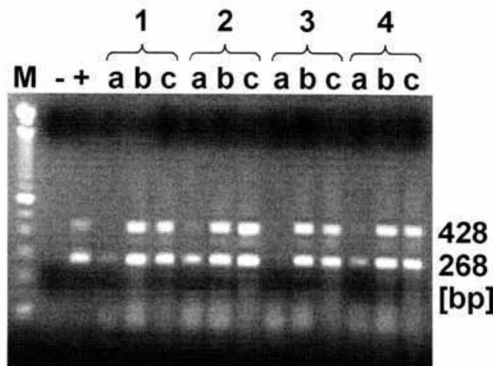

Figure 2. Comparison of PCR products (beta-globin 268 bp, N-myc 428 bp) from differently pre-treated samples, example of 4 cases (1–4): a = formalin, b = ethanol, c = frozen, + = positive control, – = negative control, M = marker (100 bp DNA ladder).

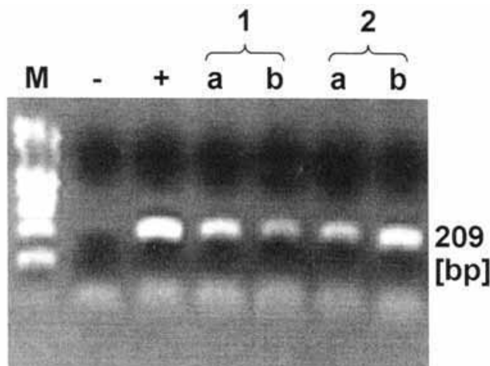

Figure 3. Comparison of "formalin-ethanol" and ethanol fixation, RT-PCR products (ALK, 209 bp), an example of 2 cases (1, 2): a = "formalin-ethanol" fixation, b = ethanol fixation, + = positive control, – = negative control, M = marker (100 bp DNA ladder).

3. No PCR products (classified as -/-): DNA in the sample was degraded to fragments smaller a 268 base pairs.

Beside the evaluation of DNA quality we performed PCR reactions, which were routinely used in lymphoma diagnostics in our laboratory: detection of IGH clonality, detection of the translocation t(14;18) in a case of follicular lymphoma and of t(11;14) in a case of mantle cell lymphoma as described previously [5, 9, 10, 14].

RT-PCR: 1 μg RNA of each sample was reversely transcribed to cDNA in a 20 μl reaction volume using 10 pmol of random hexamers as primers and 1 μl of MMLV Reverse Transcriptase (Gibco BRL). Reverse transcription included incubation period of 60 minutes at 42 °C. The products of cDNA were tested by PCRs of two ubiquitously expressed transcripts: beta-actin (95 bp) and cABL kinase (209 bp) (Tab. 1). The degree of RNA fragmentation was evaluated in a similar manner as the DNA samples: The presence of the beta-actin and ABL products was classified as +/+, +/-, or -/-.

Table 2. Comparison of PCR products from differently pre-treated samples

Sample	1. Cases +/+	2. Cases +/-	3. Cases -/-
DNA:	>428 bp	<428 bp >268 bp	<268 bp
Frozen	10/10 (100%)	0/10 (0%)	0/10 (0%)
Ethanol	10/10 (100%)	0/10 (0%)	0/10 (0%)
Formalin	4/10 (40%)	3/10 (30%)	3/10 (30%)
FEF	4/4 (100%)	0/4 (0%)	0/4 (0%)
RNA:	>209 b	<209 b >95 b	<95 b
Frozen	10/10 (100%)	0/10 (0%)	0/10 (0%)
Ethanol	10/10 (100%)	0/10 (0%)	0/10 (0%)
Formalin	0/10 (0%)	7/10 (70%)	3/10 (30%)
FEF	2/4 (50%)	2/4 (50%)	0/4 (0%)

DNA: PCR of beta-globin (268 bp) and N-myc genes (428 bp) products are classified as +/+, +/-, or -/-. RNA: RT-PCR of beta-actin (95 bp) and ABL (209 bp) RNA are compared in an analogous way. FEF – "formalin-ethanol" fixation.

Results

Control PCR and RT-PCR products from differently pre-treated samples (formalin fixed, frozen, ethanol fixed, "formalin-ethanol" fixed) were compared. The results showed, that DNA and RNA isolated from ethanol fixed samples had integrity similar to frozen samples, and considerably better than NA from the formalin fixed samples (Fig. 2, 3). According to the presence of the control PCR products we divided samples into three groups (1. +/+, 2. +/-, 3. -/-) (Tab. 2). The evaluation showed identical results in ethanol fixed samples and in frozen samples.

Even the "formalin-ethanol" fixed samples revealed a significantly better integrity of the DNA and RNA than the formalin fixed samples: all "formalin-ethanol" fixed

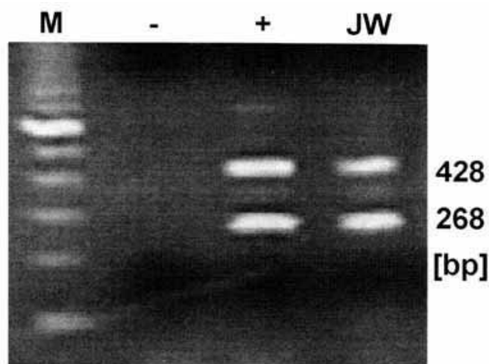

Figure 4. Clonal rearrangement of IGH, an example of 2 cases (1, 2): a = formalin, b = ethanol, c = frozen, – = negative control, p = polyclonal control, m = monoclonal control, M = marker (100 bp DNA ladder).

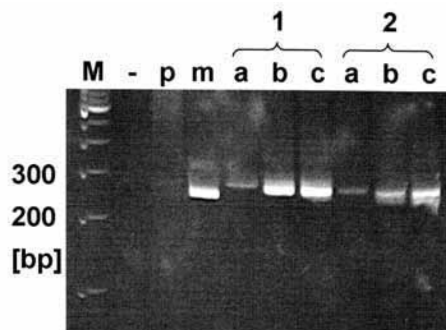

Figure 5. Good freezers may not be accessible worldwide, but the ethanol is generally ubiquitous. To emphasise universal accessibility of the proposed method, we demonstrate PCR product (beta-globin and N-myc) from a volunteer's white blood cells fixed in Johnnie Walker – Black label, 40% volume of ethanol, 12 years old (lane JW). + = positive control, – = negative control, M = marker.

samples produced PCR detectable products, the longer RT-PCR product (ABL) was missing in two samples (Tab. 2).

Evaluation of lymphoma specific PCR products was similarly enabled by the integrity of NA: all frozen and ethanol fixed samples revealed clear products of IGH rearrangement (Fig. 4), and/or translocation t(14;18) in one case of follicular lymphoma, and/or t(11;14) in a case of mantle cell lymphoma (not shown).

Discussion

The development of molecular methods stimulated a search for a fixative, which would be optimal for both approaches, morphological and molecular. Characteristics of different fixatives were studied and discussed, including ethanol [1, 2, 6, 7, 12, 13]. The advantage of ethanol fixation rests in the ability to protect nucleic acids. Shrinking cytological artefacts due to a dehydration of cells represent a major artefactual change in the tissues fixed by ethanol. To document this fact we prepared some histological and immunohistochemical slides from the ethanol fixed specimens. Some of them were suitable for the diagnostic purpose, but none was comparable with the quality of formalin fixed samples.

Finally, we realize that each fixative has some advantages and disadvantages, and the best way, how to provide tissues for both morphological and molecular diagnostics from the same surgical specimen, is to divide the samples. One portion is then fixed in buffered formalin, the other one is to be frozen. We strongly recommend to dissect and divide all samples with a potential molecular output in all laboratories, even in those, in which frozen tissue technology is not available. There is an easy alternative way, how to preserve nucleic acids in the specimens as we have shown in our study: using ethanol fixation. The only condition to introduce the method is to divide the specimens and put the portions into adequate fixatives. Ethanol as a key agent is implicitly present in every laboratory (Fig. 5).

In practice, ethanol fixation may be used in the following situations:

1) If there is a potential need of PCR diagnosis and there is no equipment for routine freezing in a pathology laboratory.

2) If the whole sample was put into formalin. Part of the sample emerged in formalin for some hours may be rescued by ethanol for the purpose of NA isolations.

3) Ethanol-fixed samples embedded in paraffin blocks may be very easily and inexpensively mailed by post. It will surpass the technical problems with mailing frozen materials. It can be appreciated especially in RT-PCR diagnostics, in which a danger of delay and thawing of the specimen (e.g. at a custom office) is eliminated.

We witness a fast development of molecular methods and their introduction to daily diagnostics. However, such a progress requires large tissue banks of appropriately handled samples. We showed that samples intended for DNA and RNA isolations may be preserved easily by the ethanol fixation. It is an alternative way how to proceed in laboratories without storage possibilities for deeply frozen tissues. This simple and effective procedure may help the patients by a more precise diagnosis, by an increasing number of investigated cases it may help us to understand and recognise the disease process.

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