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Quality assessment in diagnostic molecular pathology: experience from a German-Austrian-Swiss multicenter trial

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Abstract In order to assess the current technical standard of diagnostic molecular pathology, we have conducted a multicenter trial with 34 participating pathology laboratories in Germany, Austria and Switzerland. Formalin-fixed, paraffin-embedded tissue blocks were selected from 15 cases, comprising 4 B-cell non-Hodgkin's lymphomas, 4 T-cell non-Hodgkin lymphomas, 4 cases with lymphadenitis, 2 cases with confirmed tuberculosis and 1 case of sarcoidosis. All participating laboratories received one 10-µm section from each of the 15 cases to detect clonality using immunoglobulin heavy chain (*IgH*) gene or T-cell receptor (*TCR*)-γ gene rearrangement analysis in 12 and mycobacterial DNA in 3 cases. In addition, participants had to answer technical questions about the application of internal quality controls and performance of fragment length or sequence analysis. Correct results were reported in 80% and 90% for *IgH* and *TCR*-γ gene rearrangement analysis, respectively, and in 83% for mycobacterial DNA analysis. No significant differences in the quality of results were obvious when the individual techniques used for molecular analysis were compared. However, when two independent techniques were used by the same laboratory, a higher rate of correct results was obtained for *IgH* and *TCR* rearrangement analysis. In conclusion, this study demonstrates a high technical standard of molecular diagnostic adjuncts among the participating laboratories. Regular multicenter trials with a greater number of participating laboratories working in this field will be indispensable to ensure a continuing or increasing standard in diagnostic molecular pathology.

Key words Accreditation · Diagnostic molecular pathology · Quality assessment · Technical standard

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Introduction

More than 10 years after the first description of the polymerase chain reaction (PCR) [15, 16], molecular techniques are used not only in research laboratories, but also in clinical practice. In fact, due to its applicability for formalin-fixed, paraffin-embedded tissues, PCR-based techniques now play an important part in medical diagnostics. Amplification of tumor-type-specific DNA sequences (rearranged genes, translocations, mutations, etc.) have proved to be helpful in the differential diagnosis and characterization of several tumor types. Furthermore, gene mutations can be used for characterization of hereditary diseases and genetic susceptibility and also for "fingerprinting" of tissues. A further useful application is the amplification of species-specific gene fragments to support or to enable the diagnosis of certain infectious diseases. For the pathologist, molecular pathological approaches have become increasingly valuable diagnostic tools with enormous consequences for therapeutic procedures [2].

Among the molecular techniques most frequently applied in tumor diagnosis is the PCR-based demonstration of clonality in non-Hodgkin's lymphomas (NHL) using amplification of the *IgH* gene in B-cell lymphomas and the *TCR* genes (β or γ) in T-cell lymphomas [1, 5, 14, 23, 24]. Diagnosis of various infectious diseases can be efficiently supported by the application of molecular analysis [3, 13, 18]. For specific detection of mycobacterial DNA, amplification of the ribosomal 16S rRNA-fragment gene [6, 11], the 65-kDa antigen gene [21, 22], the insertion fragment IS986/6110 [4, 19] and the fragment MTP40 [8, 10] have proved useful. Standardization of

the molecular methods has lagged behind, however. We therefore initiated an interlaboratory quality assessment study to examine the current technical standard of frequently applied analyses in diagnostic molecular pathology. Our objective in organizing this trial was to ensure not only reproducibility and validity of molecular diagnostic analysis, but also the evaluation of quality control standards in current use.

Materials and methods

Fifteen cases were selected: 4 B-cell NHLs, 4 T-cell NHLs, 4 cases of lymphadenitis, 2 cases of confirmed tuberculosis and 1 case of sarcoidosis. The pathohistological classification of the lymphomas was carried out in accordance with the defined histopathological criteria [9], based upon 5-µm sections stained with hematoxylin-eosin or Giemsa and immunohistochemically stained slides. All cases had been previously characterized for the *IgH* gene rearrangements (IgH group) and *TCR-γ* gene rearrangements (TCR group) according to published protocols [14, 24], revealing monoclonality in all the neoplastic cases and a polyclonal pattern in all the nonneoplastic cases (Table 1).

The *IgH group* was composed of 6 cases: 2 nodal B-cell NHLs (1 mantle-cell lymphoma and 1 marginal zone NHL), 2 extranodal B-cell NHLs (1 mantle-cell lymphoma of the soft tissue and 1 diffuse large B-cell NHL of the femur) and 2 polyclonal controls cases (1 lymph node with a polymorphic Epstein-Barr virus-associated lymphoproliferation and 1 reactive lymph node with prominent follicular hyperplasia).

The *TCR group* was also composed of 6 cases: 2 nodal T-cell NHLs (1 of Sézary syndrome and 1 of mycosis fungoides in lymph nodes) and 2 extranodal T-cell NHLs (1 peripheral T-cell NHL of the spleen and 1 peripheral T-cell NHL of the skin). Also, in this group 2 cases of nonneoplastic T-cell proliferation (1 lymph node affected by a reactive lymphadenitis and 1 lymph node affected by Kikuchi's disease with expansion of the interfollicular zone) were used as polyclonal controls.

The *TBC group* contained 3 cases: 1 lymph node with a caseating granulomatous lymphadenitis and 1 ovary with extensive granulomatous, partially caseating inflammation, both positive for DNA of *Mycobacterium tuberculosis* and 1 lymph node with a typical histological pattern of sarcoidosis with a noncaseating granulomatous inflammation, which was negative for DNA of typical and atypical mycobacteria, using different PCR protocols [3, 12, 17]. All cases were negative for acid-fast bacilli according to Ziehl-Neelsen staining. In the 2 positive cases in the TBC group, clinical follow-up data were available, e.g. response to TBC-specific therapy. Table 1 summarizes the histopathological and molecular data of the 15 cases selected. All institutes of pathology in Germany, Austria and Switzerland that were registered with the "Deutsche Gesellschaft für Pathologie" and the "Berufsverband deutscher Pathologen" (873 institutes) were asked to take part in this quality assessment trial for molecular diagnosis. Overall, 44 laboratories intended to participate and received 15 tissue sections each, taken from routinely processed formalin-fixed and paraffin-embedded surgical specimens. For each case, an Eppendorf tube containing a single (unmounted) 10-µm section was sent to each of the laboratories. The average size of the tissue sections used was approximately 1 cm^2, and each consisted mainly of tumor and granulomatous tissue, respectively. Mounted tissue sections for histopathological examination were not sent to the participants.

Furthermore, the participants received a letter with a list of the cases, briefly explaining the rules of this trial: we asked the participants whether clonality in the cases suggestive of lymphoma in the IgH and in the TCR group could be proven and whether mycobacterial DNA could be detected in the TBC group. We did not communicate the histopathological diagnosis. In addition, the participants were asked for the DNA concentration per sample, and also whether a housekeeping gene was used for DNA quality control. Furthermore, a report on the gene regions examined, the length of the PCR products (base pairs) and whether fragment length analysis or sequencing was used or both was requested of the participants.

Results

Response and quality controls

Of the 44 participants, 34 (77%) returned the questionnaire filled in completely, whereas the remaining 10 participants (23%) did not return the questionnaire. Of the 34

Table 1 Final histopathological and molecular data of the 15 cases employed in the quality control assessment (*EBV* Epstein-Barr virus, *NHL* non-Hodgkin's lymphoma)

Case no.	Tissue localization	Histopathological diagnosis	Molecular diagnosis
Cases for IgH rearrangement analysis			
1	Lymph node	EBV-associated lymphoproliferation	Polyclonal
2	Lymph node	Mantle cell lymphoma	Monoclonal
3	Soft tissue	Mantle cell lymphoma	Monoclonal
4	Lymph node	Reactive follicular hyperplasia	Polyclonal
5	Bone	Diffuse large B-cell NHL	Monoclonal
6	Lymph node	Marginal zone B-cell NHL	Monoclonal
Cases for TCR rearrangement analysis			
1	Lymph node	Reactive lymphadenitis	Polyclonal
2	Lymph node	Sézary syndrome	Monoclonal
3	Skin	Peripheral T-cell NHL	Monoclonal
4	Lymph node	Kikuchi lymphadenitis	Polyclonal
5	Spleen	Peripheral T-cell NHL	Monoclonal
6	Lymph node	Mycosis fungoides	Biallelic rearrangement
Cases for detection of mycobacterial DNA			
1	Lymph node	Noncaseating granulomatous inflammation	Negative for mycobacterial DNA
2	Ovary	Caseating granulomatous inflammation	Positive for DNA of *M. tuberculosis*
3	Lymph node	Caseating granulomatous inflammation	Positive for DNA of *M. tuberculosis*

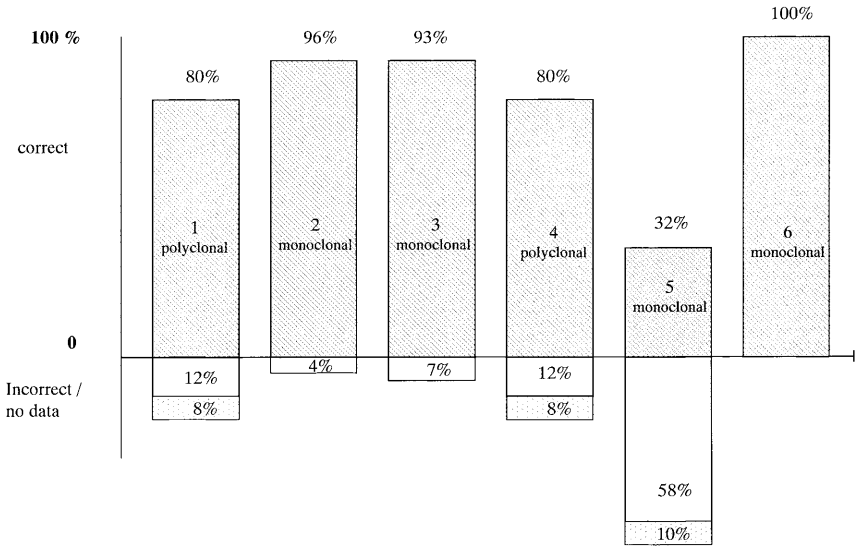

Fig. 1 Results of the IgH gene rearrangement analysis: correct (*hatched*) incorrect (*blank*) and no data (*spotted*)

responders, 30 (88%) were located in Germany, 2 (6%) in Switzerland and 2 (6%) in Austria. The 30 German participants were 19 (64%) university institutes, 7 (23%) quite large institutes connected with nonuniversity hospitals and 4 (13%) private laboratories. All the Swiss and Austrian participants were university institutes.

There was a large difference in the range of DNA concentration reported by the various laboratories (0.08–400 µg), but no correlation was detected between the amount of extractable DNA and the quality of the final results of the molecular analysis. Internal quality controls were performed by 19 (56%) of the 34 participants with the aid of amplifying housekeeping genes: 8 laboratories amplified the beta globin gene, 4 the beta actin gene, 3 the glyceraldehyde-3-phosphate-dehydrogenase gene, and 1 each the *APC* gene, the *HRS1* gene, the *factor V* gene and the *D2S123* microsatellite locus.

Some of the participants (29%) conducted a fragment length analysis using an automatic system to support the diagnosis of mono- or polyclonality in both the IgH and the TCR groups. Two institutes (6% of the participants) tested their PCR products by sequencing using an automated sequence analyzer (in 1 case to confirm the results of the clonal analysis for the cases of the IgH group, and in the second one by subtyping the mycobacteria). In general, in none of the three groups were the results influenced by the type of primer pairs used for PCR or by whether agarose gel or fragment length analysis was performed to detect the PCR products.

IgH analysis

Thirty-one participants (91% of the 34 who returned the questionnaire completely filled in) conducted the analysis of IgH rearrangement. Overall, 80% of all of the IgH results (average) were correct (Fig. 1).

Fifty-eight percent of the participants conducted IgH rearrangement analysis employing a semi-nested PCR, and 30% a single-step PCR, while 6% used both methods; another 6% of the participants did not report the method applied. No noticeable correlation was found between the procedures applied and the results obtained (15% of the results were incorrect with each method). Only when both techniques were used the rate of errors was reduced to 8%.

Sixty-two percent of the participants examined framework regions (FR), 3 that of the *IgH* gene only, 29% the FR2 and FR3 regions, and 6% the FR1 and FR3 regions; 1 participating laboratory did not disclose the region at all. In laboratories in which two FRs were amplified a lower percentage of errors (9%) was evident than in those that examined only one region (16%). Furthermore, the single laboratory in which the region examined was not disclosed had a rather high error rate of 30%. The high percentage of incorrect results in case 5 could be explained in part by the presence of a high number of reactive lymphocytes mixed with a single dominant (neoplastic) clone. This was demonstrated by cloning and sequencing of PCR products in this particular case.

TCR analysis

In 26 (77%) of the 34 participants who returned the questionnaire rearrangements of the *TCR-γ* gene were analyzed. Overall, 90% of all of the TCR results (average) were correct (Fig. 2). In 70% of the participating laboratories the analysis was conducted with a single-step PCR, in 15% with a semi-nested PCR, and in 8% by two methods (single-step and semi-nested or semi-nested and nested PCR), while 7% did not disclose the technique used. Those participants who employed two techniques obtained 100% correct results, whereas those who used only one method obtained 8% of false results. The percentage of error rose to 30% for the participants who did not disclose the method of analysis used.

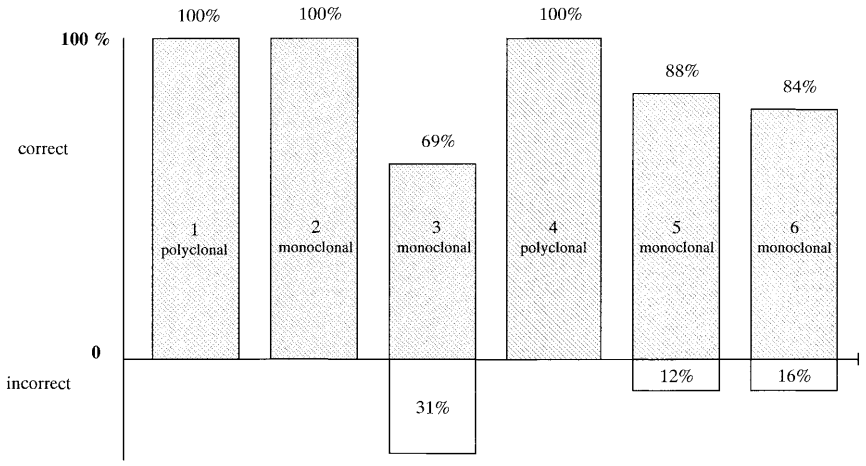

Fig. 2 Results of the *TCR-γ* gene rearrangement: correct (*hatched*) and incorrect (*blank*) results

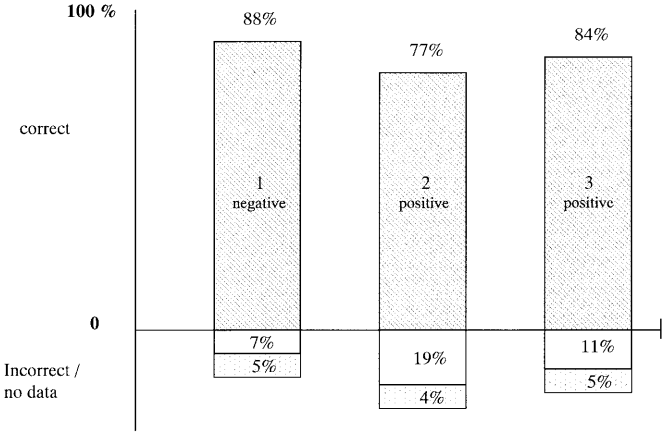

Fig. 3 Results of mycobacterial DNA detection: correct (*hatched*) and incorrect (*blank*) results and no data (*spotted*)

TBC analysis

Twenty-six (77%) of the 34 participants who returned the questionnaire conducted PCR analyses to detect mycobacterial DNA. Overall, 83% of all of the TBC results (average) were correct (Fig. 3). Twenty percent of the participants tested for the presence of mycobacterial DNA with a single-step PCR, 19% with a semi-nested PCR, and 34% with a nested PCR; 12% employed two techniques (single-step and semi-nested PCRs or semi-nested PCRs and nested PCRs), while 15% of the participants did not disclose the method used. Fifty-seven percent of the participants examined the presence of mycobacterial DNA by amplifying the insertion segment *IS6110* gene (also known as *IS986*), 7% the gene which codes the 65-kDa antigen, and 7% examined both; 4% analyzed the last two gene regions together with the gene fragment MTP40; 7% amplified the gene that encodes the mycobacterial 16 ribosomal RNA, while 18% did not disclose which region of the gene was examined. No significant differences in results were found between the different techniques employed or the gene regions examined.

Discussion

The results of this quality assurance study for diagnostic molecular pathology provide clear evidence that even with a single 10-µm section of paraffin-embedded tissue it is possible to obtain excellent results, in terms of both amplification of the desired gene regions and reproducibility of the data reported. In some cases in the IgH and the TCR groups, the results were even 100% concordant. These findings prove the validity of the PCR techniques in routine diagnostic work on paraffin-embedded tissues and ensure that the results can be reproduced in different laboratories.

It should be emphasized that our study was designed to assess the technical standard of molecular methods only, since the participants received one unmounted paraffin section for each case and a clear (yes/no) differential diagnosis.

The quality of the whole diagnostic procedure, including histomorphology and, if necessary, immunohistochemistry, was not evaluated by this approach. Therefore, the false-negative or false-positive results obtained in some of the cases only reflect a failure of the molecular technique applied or the misinterpretation of the results.

Among the cases of the IgH group, 1 case (no. 5, Fig. 1) revealed a low percentage (32%) of correct answers among those returned. Cloning and successive sequencing of the PCR product had shown the simultaneous presence of a strong component of reactive background lymphocytes responsible for a high number of pseudoclonal bands, which might have masked the single specific band of the neoplastic cells, thus leading to a false-negative interpretation. The difficulties in the separation of a clonal population of tumor cells against a strong reactive background are well known from the literature [19, 24]. In cases 1 and 4, 12% of the participants produced false-positive results: this may be explained by the high number of germinal centers with reactive lymphocytes present in the environment of the neoplastic B-cell proliferations. Thus, preferential amplification of non-neoplastic clones of mitotically active reactive lymphocytes may have produced a “clonal status.”

Although the results of the TCR group are excellent from an overall point of view, it must be borne in mind that 30% of results were false-negative in case 3. In this case the amplification of the reactive T-lymphocyte component, associated with neoplastic cells, had caused false-negative interpretations. Providing an additional HE-stained section of this peripheral T-cell lymphoma could possibly have avoided these false-negative results: the evidence of reactive lymphoid tissue intermingled with the neoplastic T-cell proliferation might have led participants to exercise caution in interpreting the PCR results.

Within the TBC group the two positive cases revealed 11% and 19% false-negative results. The reason for the failure of amplifying mycobacterial DNA from these specimens may be that isolating DNA from mycobacteria requires harsh treatment to open the rigid walls of these bacteria [7]. In case 1, however, it could be discussed whether the 7% false-positive results were due to a contamination during the procedures of DNA isolation or preparation of PCR.

A relatively high percentage of the laboratories (44%) did not subject the extracted DNA to an internal quality test, such as the amplification of a housekeeping gene, which could have confirmed a good amplifying capability. Those laboratories that did not indicate primers used for PCR or the region of the gene examined (especially of the *IgH* gene), and those that did not state the PCR method applied (especially for detecting *TCR* gene rearrangement) came up with a relatively high rate of false results. Nevertheless, our overall data highlight an excellent result for this first trial in molecular diagnostic pathology in the participating countries.

The application of the medical techniques employed in clinical diagnosis requires standardization. Regular quality assessment in molecular pathology as described here could lead step by step to an indispensable accreditation system used by all laboratories working in this field. Such an accreditation system can provide additional security for patients and for those institutions (e.g. insurance companies) that will have to cover the charges for molecular analysis. Our trial suggests that experienced laboratories can be encouraged to use PCR analysis in diagnostic pathology. The methods are fast and highly efficient. However, the final diagnostic interpretation can and should only be done when it is closely linked with histopathology. Only in this case will molecular pathology help to improve the quality of diagnosis in histopathology.

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